UNIVERSITY OF ALBERTA

# BIOLOGICAL SIGNIFICANCE OF FK506 METABOLITES

By

MING-CHIH LEE ©

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**.

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# Abstract

FK506 (Tacrolimus) is an immunosuppressive agent generally used in solid organ transplant patients to prevent rejection. The major mechanism of FK506 is through inhibition of calcineurin that leads to the decreased production of interleukin-2 (IL-2). IL-2 is an important factor that promotes the proliferation of T-lymphocytes when the immuno-response occurs.

Although scientists have determined a therapeutic range for FK506 in patients' trough blood to maintain the effect of FK506, many patients still undergo chronic rejection or side effects. These may result from an unknown mechanism of FK506 itself or from the influence of FK506 metabolites. In addition, the analytical methods used to monitor FK506 blood concentrations may have a positive bias due to the nature of the methods.

In order to study the significance of FK506 metabolites, the patterns of these metabolites formed from various biological sources were compared. A rabbit liver microsomal metabolic system was chosen to produce large quantities of FK506 metabolites. The significance of FK506 metabolites was studied. Mixed lymphocyte reaction and the inhibition of calcineurin were used to study immunosuppressive activity. An enzyme-linked immunosorbent assay (ELISA) was used to investigate cross-reactivity. Pig aorta endothelial cells were used to study the influence of FK506 and its metabolites on the release of the endothelin and prostacyclin since it was thought that the change of renal filtration rates produced by those substances was the cause of renal toxicity.

13-Demethyl FK506; 15-demethyl FK506; 13,31-didemethyl FK506; and demethyl-hydroxy FK506 (peak no. 4) were consistently found in patients' blood in addition to FK506. 13,15-didemethyl FK506; hydroxy-FK506 (peak no. 2); and hydroxy-FK506 (peak no. 3) were sometimes present in human blood. Dihydroxy FK506 was only seen in human urine, not human blood. 31-Demethyl FK506 was not found in patients' blood or urine, although it has the same potency of immunosuppression as FK506 and has over 100% cross-reactivity with anti-FK506 antibody in ELISA. 15-Demethyl and 15,31-didemethyl FK506 have 90% cross-reactivity. As for the rest of the FK506 metabolites, they do not show any significant immunosuppression or cross-reactivity. 31-demethyl; 13,31-didemethyl; and 15,31-didemethyl FK506 were found to decrease the growth rate of the endothelial cells. The hypothesis for the possible mechanism of renal toxicity was unproven. Further investigations on possible involvement of FK506 metabolites in renal toxicity are warranted.

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# TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Chapter I Introduction ---------------------------------------------------------------------1

Preface ------------------------------------------------------------------------------1

Concept of Rejection ------------------------------------------------------------2

Immunosuppression-------------------------------------------------------------8

FK506------------------------------------------------------------------------------11

Mode of Action -------------------------------------------------------------------12

Pharmacokinetics ----------------------------------------------------------------14

Metabolism and Elimination ---------------------------------------------------16

Side Effects -----------------------------------------------------------------------21

Therapeutic Drug Monitoring of FK506 --------------------------------------27

Liquid Chromatography/Mass Spectrometry --------------------------------29

Problems with FK506 Immunosuppressive Therapy ----------------------33

Objectives and Rationale -------------------------------------------------------35

Chapter II Identification and purification of FK506 metabolites ------------------39

Objective --------------------------------------------------------------------------39

Materials and Methods ----------------------------------------------------------39

Purification of FK506 from pharmaceutical capsules -------------------39

Preparation of acetyl-FK506 as an internal standard --------------------41

Development of LC/MS method to monitor FK506 and possible metabolites -----------------------------------------------------------------43
Extraction of FK506 metabolites from human blood specimens -------45
Extraction of FK506 metabolites from human urine specimens --------46
*In vitro* generation of FK506 metabolites --------------------------------47
Investigation of possible phase II FK506 metabolites from human urine specimens ---------------------------------------------------------------50
Results -------------------------------------------------------------------53
Chapter III Comparison of methods used to monitor trough FK506 blood concentrations ---------------------------------------------------------------67
Objective ---------------------------------------------------------------67
Materials and Methods -------------------------------------------------68
FK506 enzyme linked immunosorbent assay (ELISA) -----------------68
FK506 IMx micro-particle immunoassay (MEIA) ---------------------70
LC/MS assay ----------------------------------------------------------72
Comparison -----------------------------------------------------------74
Results ---------------------------------------------------------------75
Chapter IV Biological significance of FK506 metabolites -----------------------87
Objective ---------------------------------------------------------------87
Materials and Methods -------------------------------------------------88
LC/MS assay to measure the steady state concentration of FK506 metabolites in patients' trough blood ------------------------------------88
Two-way mixed lymphocyte reaction ------------------------------------90

Cross- reactivity study --------------------------------------------------------93

Calcineurin (CN) assay --------------------------------------------------------94

Culture of pig aorta endothelial cells ------------------------------------101

$^{125}$I radioimmunoassays for endothelin 1,2 and 6-keto-prostaglandin $F_{1\alpha}$ ------------------------------------------------------------------------104

Results -----------------------------------------------------------------------108

Chapter V Discussion ----------------------------------------------------------121

Identification of FK506 Metabolites ---------------------------------------121

Therapeutic Monitoring of FK506 -------------------------------------------123

Biological Significance of FK506 Metabolites ---------------------------128

Metabolite patterns and trough blood steady state concentrations ----128

Immunosuppressive activity ---------------------------------------------129

Cross-reactivity ----------------------------------------------------------131

Influence on the release of vaso-active substances from endothelial cells ------------------------------------------------------------------------133

Biological significance ----------------------------------------------------136

Possible Directions for Further Investigation -----------------------------140

Conclusions -------------------------------------------------------------------145

References ---------------------------------------------------------------------151

# LIST OF FIGURES

Figure I-1: Chemical structure of FK506 and its metabolites---------------------38

Figure II-1: Electrospray mass spectrum of purified FK506 ----------------------57

Figure II-2 A: LC/MS profile of acetyl-FK506 and FK506 ----------------------58

Figure II-2 B: Chemical structure of acetyl-FK506--------------------------------59

Figure II-3: Electrospray mass spectrum of purified acetyl-FK506 -------------60

Figure II-4: LC/MS profiles of FK506 and its metabolites from different biological sources ----------------------------------------------------------------61

Figure II-5: Electrospray mass spectrum of purified demethyl FK506 species ------------------------------------------------------------------------------------62

Figure II-6: Electrospray mass spectrum of purified didemethyl FK506 species ---------------------------------------------------------------------------------------63

Figure II-7: LC/MS profile of morphine/morphine glucuronide in urine before and after the enzymatic reaction of β-glucuronidase/sulfatase -------------------64

Figure III-1: Representative calibration curve of FK506 in human whole blood measured by LC/MS assay ---------------------------------------------------------82

Figure III-2: Linear regression for the results of FK506 trough blood concentrations measured between LC/ MS and ELISA ---------------------------83

Figure III-3: Bias plot between ELISA and LC/MS ------------------------------84

Figure III-4: Linear regression for the results of FK506 trough blood concentrations measured between MEIA and ELISA -----------------------------85

Figure III-5: Bias plot between MEIA and ELISA --------------------------------86

Figure IV-1: Results of the studies on the influence of FK506 and its metabolites on the release of endothelin-1,2 and prostacyclin from the culture of pig aorta endothelial cells --------------------------------------------------------------------113-120

Figure V-1: Flow chart of the relationship between heat shock proteins, interleukin-1, interleukin-6, endothelin-1,2, and prostaglandin $I_2$ ---------------150

# LIST OF TABLES

Table II-1: Summary of FK506 metabolite patterns from various biological sources --------65

Table II-2: t- test results of the investigation of possible phase II FK506 metabolites --------66

Table III-1: Calibration curve summary for FK506 in human whole blood -----78

Table III-2: Between-run precision and accuracy of LC/MS assay --------79

Table III-3: Within-run precision and accuracy of LC/MS assay --------80

Table III-4: Extraction efficiencies of FK506 and acetyl- FK506 from human whole blood --------81

Table IV-1: Steady state concentrations, immunosuppression, and cross-reactivity of FK506 and its metabolites --------111

Table IV-2: Results of calcineurin inhibition (whole blood vs. peripheral blood mononuclear cells) and the mixed lymphocyte reaction of FK506 and its metabolites --------112

Table V-1: Published FK506 metabolites cross-reactivities and immunosuppression --------148

Table V-2: Clinical Significance Indices of FK506 and its metabolites --------149

## LIST OF ABBREVIATIONS

| | |
|---|---|
| aa | amino acid |
| ACN | acetonitrile |
| APC | antigen-presenting cells |
| ATP | adenosine triphosphate |
| AUC | area under the curve |
| $B_0$ | zero standard |
| BME | β-mercaptoethanol |
| BSA | bovine serum albumin |
| $CaCl_2$ | calcium dichloride |
| CD4 | cluster designation 4 |
| CD8 | cluster designation 8 |
| CDK | cyclin dependent kinase |
| CMV | cytomegalovirus |
| CN | calcineurin |
| Co. | corporation |
| CSI | clinical significant index |
| CTL | cytotoxic T lymphocyte |
| CV | coefficient of variation |
| CYP | cytochrome P450 |
| DC | direct current |
| DTT | dithiothreitol |
| EBV | Epstein Barr virus |

| | |
|---|---|
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol-bis (β-aminoethylether) |
| ELISA | enzyme- linked immunosorbent assay |
| ET | endothelin |
| FKBP | FK506 binding protein |
| µg | microgram |
| µM | micromolar |
| ×g | relative centrifugal force |
| GSF | granulocyte stimulation factor |
| GMCSF | granulocyte monocyte colony-stimulation factor |
| HCl | hydrogen chloride |
| HLA | human lymphocyte antigen |
| HPLC | high performance liquid chromatography |
| ICAM 1 | intercellular adhesive molecule 1 |
| IFNα | interferon α |
| IFNγ | interferon γ |
| IgG | immunoglobulin G |
| IgM | immunoglobulin M |
| IL-1 | interleukin-1 |
| IL-2 | interleukin-2 |
| IL-2 R | interleukin-2 receptor |
| IL-4 | interleukin-4 |

| | |
|---|---|
| IL-6 | interleukin-6 |
| $IP_3$ | inositol 1,4,5-triphosphate |
| $IP_3R$ | inositol 1,4,5-triphosphate receptor |
| L | liter |
| LC/MS | liquid chromatography/mass spectrometry |
| LFA-1 | lymphocyte- function- associated antigen 1 |
| M | molar |
| MCSF | macrophage colony-stimulating factor |
| MEIA | micro-particle enzyme immunoassay |
| MeOH | methanol |
| MHC | major histocompatibility complex |
| MgCl2 | magnesium dichloride |
| mL | milliliter |
| MLR | mixed lymphocyte reaction |
| 6-MP | 6-mercaptopurine |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| NF-AT | nuclear factor of activated T lymphocyte |
| NO | nitric oxide |
| NSB | non-specific binding |
| PBMC | peripheral blood mononuclear cell |
| $PGI_2$ | prostaglandine $I_2$ |
| PMSF | phenylmethylsulfonyl fluoride |

| | |
|---|---|
| PP1 | phosphatase 1 |
| PP2a | phosphatase 2a |
| PP2c | phosphatase 2c |
| QC | quality control |
| RBC | red blood cell |
| RF | radiofrequency |
| RIA | radioimmunoassay |
| TC | total counts |
| TDM | therapeutic drug monitoring |
| TFA | trifluoroacetic acid |
| TGFβ | transforming growth factor β |
| TNFα | tumor necrosis factor α |
| TxA2 | thromboxane A2 |
| UDP | uridine disphosphate |
| UV | ultraviolet |
| Vd | volume of distribution |

# Chapter I

## Introduction

### Preface

Organ transplantation has evolved from experimental procedures to commonly used therapeutic options during the past century. Tens of thousands of people around the world have benefited from this type of procedure and lived years beyond the natural history of their diseases. However, allograft rejection, an immunologic process which occurs naturally when foreign organs are transplanted into recipients' bodies, ranks second in importance only to the shortage of transplantable organs in terms of limitations to further progress for transplantation. In 1908, Benjamin and Sluka observed that total body irradiation of rabbits impaired the synthesis of antibodies toward bovine serum (Flye, 1989). Another finding, that irradiation slowed the development of immunity toward tumor allografts, was revealed six years later (Flye, 1989). In 1914 and 1916, it was discovered that two simple compounds, benzene and toluene, had immunosuppressive effects (Kolbeck et al., 1992). In 1952, it was documented that the combination of methyl-bis-[β-chloroethyl]-amine hydrochloride (nitrogen mustard), corticosteroids, and splenectomy prolonged graft survival of mongrel canine allografts (Kolbeck et al., 1992). The finding that anti-leukemic drug 6-mercaptopurine (6-MP) could prevent rabbits from producing antibodies to many antigenic stimuli initiated the modern era of pharmacological immunosuppression (Medawar, 1944; Flye, 1955). The discovery and development of various immunosuppressive drugs to prevent the allograft rejection have further increased the survival rate of transplant patients. These drugs have also led to significant investigations in attempting to elucidate immunologic pathways causing allograft rejection and autoimmune disorders and reactions to infectious diseases.

Despite the rapid progress in the improvement of immunosuppressive therapy, the less obvious indirect adverse effects of transplant rejection are still of considerable scope and importance. These indirect effects arise primarily from the complication of clinical attempts to control rejection and include a variety of infections, drug toxicity, lymphoproliferative disorders and malignancies (MacGregor et al., 1995). Continued development of different types of therapies and further understanding of the existing immunosuppressants are required to overcome these obstacles. The focus of my thesis is on one of the existing immunosuppressive drugs, namely FK506 (Tacrolimus, Prograf®).

## Concept of Rejection

The rejection happening in transplantation is often characterized as a two-phase process involving host recognition followed by a complex immune response. This results in various degrees of reversible or irreversible allograft damage.

The host recognition (afferent sensitization) phase depends on the expression of immunogenic donor target antigens in transplanted tissue and the interaction of host (or donor) antigen-presenting cells (APCs) with effector cells (primarily lymphocytes and macrophages) capable of both identifying and responding to these antigens (Stadlbauer et al., 1997). Cell surface antigens capable of eliciting a host rejection response include the Human Lymphocyte Antigen (HLA) system, ABO and Lewis blood group antigens, non-HLA monocyte or endothelial cell antigens, and possibly other tissue-specific non-HLA antigens (Braun, 1988; Pattison et al., 1997). These antigenic systems vary among species and among different organs and cells within the same species. In general, the HLA and ABO blood group antigens are the most widely distributed and significant determinants for allograft rejection (Bollinger et al., 1989). The HLA molecules on cell surfaces are important in the defending system (immunity) because they bind foreign antigens from microbes and present them to T cells for potential immunologic recognition and activation. Before antigen becomes associated with HLA molecules, it undergoes processing or degradation, often within the cell that is to express the antigen. The HLA system exhibits extensive diversity within several different series of cell surface antigens divided into two major classes: Class I (HLA-A, B, and C) and class II (HLA-DR, DQ, and DP) (Kolbeck et al., 1992; Pattison et al., 1997). Class I HLA antigens are present on essentially all nucleated cells while class II HLA antigens under normal circumstances are expressed primarily on B-lymphocytes, antigen-presenting cells such as macrophages, dendritic cells, and certain other specialized epithelial and endothelial cells. It is believed that HLA polymorphism is the result of the evolutionary advantage conferred on the species by a diverse antigen-processing system capable of interacting with the widest possible range of invading microbes. Because of the HLA polymorphism within the species, the foreign HLA molecules themselves or HLA bound to processed donor antigen are immunogenic and serve as the targets for destruction by host immune cells sensitized to these foreign alloantigens. Hence, clinical HLA typing prior to transplant surgery has shown to be very useful in optimizing donor selection for potential recipients to avoid rejection (Kolbeck et al., 1992; Pattison et al., 1997). However, there is usually only a short time between the organ becoming available and finding suitable recipients. Only microlymphocytotoxic tests to identify serologically defined HLA-A, B, and DR antigens are routinely applied at present for potential recipients of organs. This results in a limited ability to match donors' HLA with recipients', and prevents the identification of certain mismatched antigens. This is one of the reasons why chronic rejections will occur on patients under immunosuppressive therapy. The influence of the blood group antigens on the organ rejection will be discussed later.

Normally, the process of immune recognition and sensitization is a prerequisite to the allograft rejection response. Antigen-presenting cells (APCs) and T-lymphocytes (both helper and cytotoxic) play the leading roles in the immune recognition (Kolbeck et al., 1992; Stadlbauer et al., 1997). When transplantation occurs, the rate and pattern of lymphocyte migration is disrupted.

This results in an increase in the influx rate of white blood cells into the graft (Kupiec-Weglinski et al., 1985; Nemlander et al., 1982; Olszewski et al., 1987) and is determined by specialized capillary endothelium within the graft (Butcher et al., 1980). The regulation of specific endothelial and lymphocyte receptors responsible for tissue-specific homing of lymphocytes is a crucial component in the rejection response (Bierer et al., 1988; Jalkanen et al., 1986; Woodruff et al., 1987). Specific receptor-ligand pairs such as intercellular adhesive molecule 1 (ICAM 1) and lymphocyte-function-associated antigen 1 (LFA-1), primarily expressed on lymphocytes or myeloid cells, demonstrate enhanced tissue expression during acute allograft rejection (Adams et al., 1989; Jutila et al., 1989). Lymphocytes infiltrating a rejecting allograft become specifically sensitized to the mismatched donor alloantigens present on the cells of grafted tissue. A minority of these lymphocytes proliferates within the graft (Ruers et al., 1990). Alloantigen is also shed from the graft and makes its way to regional lymph nodes and the spleen, where additional sensitization can occur (Emeson, 1977; Livnat et al., 1976). Host spleen and other lymphoid tissues are probably the primary sites of allosensitization (Larsen et al., 1990).

Subsequent to sensitization by appropriate antigen and co-stimulator, T lymphocytes are activated and undergo a series of tightly regulated biochemical and structural changes which result in lymphocyte proliferation, a variety of effector functions, and immunologic memory. At least seventy molecules are involved with the activation and proliferation stages. One of these molecules, Interleukin-2 (IL-2), serves as an important regulator to oversee the entire process (Crabtree, 1989). IL-2 is often referred to as an autocrine molecule because it is both produced and utilized by T lymphocytes. Following immunoactivation, IL-2 and IL-2 receptor (IL-2 R) genes are expressed by T lymphocytes (Taniguchi et al, 1986). The binding of IL-2 to high-affinity IL-2 R together with other co-stimulation in T lymphocytes lead to a series of intracellular events and result in the activation and proliferation of effector cells.

The two major effector arms of the immune system are antibody (humoral immunity) and immune cells (cell-mediated immunity). The immune cells include antigen-specific and nonspecific lymphocytes, monocytes, and granulocytes. There is extensive interdependence and redundancy between the formation, regulation, and function of these two effector divisions. In general, it is believed that allograft rejection involves allospecific effectors manifested primarily by antibodies and T lymphocytes, although there is evidence that all aspects of the immune system can participate in the rejection reaction (Kolbeck et al., 1992). The group of ABO blood antigens is an example of alloantigens involving hyperacute or accelerated acute, antibody-mediated rejections. The presence of a high titer of IgM isohemagglutinins against A or B blood group antigens expressed normally on the vascular tissue of donor organs often leads to severe rejection reactions. The ability of Lewis blood group antigens to adhere to vascular endothelium in secretion-positive patients has also been suggested to stimulate rejection in renal allograft recipients, although this correlation remains unclear at present. Thus, preformed antibodies capable of destroying alloantigen-laden donor tissue must be screened for by cross-matching tests prior to transplantation. In addition to preformed antibodies, directed complement fixation leading to lysis of graft cells, antibody-dependent cellular cytotoxicity, and possible alloantibody enhancement or induction of allograft or inflammatory cell cytokine expression can also lead to destructive antibody-mediated rejection. The phenotypes of the T lymphocytes most vital to the cell-mediated rejection include CD4+ [Major Histocompatibility Complex (MHC) class II reactive] and CD8+ (MHC class I reactive). Each has overlapping or mutually exclusive effector roles, depending on the phase of rejection (Braun, 1993; Rosenberg et al., 1991) and the nature of the antigen stimulus (Hall, 1991; Krensky et al., 1990; Sablinski et al., 1991). The fine details of how allosensitized immune effector cells actually cause allograft dysfunction or destruction are not completely understood. The most likely scenario for effector function following allosensitization involves infiltration and expansion of graft T lymphocytes and macrophages, which work in alliance to destroy donor cells bearing MHC or minor alloantigens. The inflammatory cytokine network made up of small-molecule glycoproteins serves vital communication and effector functions in rejection (Arai, 1990). This system includes not only the important regulatory cytokines such as IL-1, IL-2, IL-4, IL-6, IFNγ, TNFα, and macrophage colony-stimulating factor (MCSF) (Bugeon et al., 1992; Dallman et al., 1991; Ford et al., 1990; Ford et al., 1991; Wu et al., 1992) but also the cytotoxic enzymes or toxins produced by cytotoxic T lymphocytes (CTLs) (Lipman et al., 1992; Mueller et al., 1993). Other immune cells such as monocytes, neutrophils, and eosinophils contribute to the allograft tissue damage that results from rejection

(Adams et al., 1990). These inflammatory cells also have major impacts on the normal homeostatic balance of a variety of physiologic endogenous systems within the allografts such as coagulation (Szabo et al., 1990) and eicosanoid synthesis (Lewis et al., 1990; Post et al., 1991).

The current methods to induce allograft acceptance primarily involve deletion of alloreactive T lymphocytes through nonspecific immunosuppressive agents (Burnet et al., 1949). Attempts to induce a state of self-tolerance to the foreign HLA antigens are also at experimental stages. The problem of developing tolerance to transplanted tissue is that the particular nonself HLA antigens are not present in the fetal thymus (the locus to form self-tolerance). The approaches that people have tried to induce tolerance focus on the tolerance of peripheral T lymphocytes (Ramsdall et al., 1990). These include the induction of a state of specific inactivation or anergy of the T lymphocytes by occupying T cell receptors in the absence of a costimulatory signal from splenetic antigen-presenting cells (Burkly et al., 1990; Nossal, 1989; Ramsdall et al., 1989; Schwartz, 1990; Sprent et al., 1990) and the induction of lymphoid cells that are capable of negative regulation or suppression of alloreactive T lymphocytes (Burdick, 1992).

## Immunosuppression

The management of immunosuppression is one of the greatest challenges for transplant physicians. Insufficient immunosuppression may result in rejection that threatens the transplanted tissue and patient survival, while excessive immunosuppression increases the risk of infection and malignancy. Variable responses of the individual patients, multiple environmental influences, drug-related complications, drug interactions, and patient compliance demand constant monitoring and adjustments of the immunosuppressive regimen. Unfortunately, some adverse effects such as hypertension and headache must be accepted and coped with by the patients, and should not prompt dosage reduction, as maintenance of adequate immunosuppression is crucial to long-term allograft and patient survival (Costanzo, 1994).

Over the past ten years, methods of immunosuppression have evolved from the use of two immunosuppressive drugs (double therapy) to that of three drugs (triple therapy). In the early times, patients were given high dose oral corticosteroids and azathioprine as post-transplant therapy. Corticosteroids are anti-inflammatory drugs (Kull, 1988; Rugstad, 1988) that inhibit gene transcription of several cytokines (Wielckens et al., 1987). Thus, antigen-presenting macrophages cannot release interleukin-1 (IL-1) and interleukin-6 (IL-6) (Waage et al., 1990) which provide a signal to activated T-lymphocytes for IL-2 receptor expression. Without IL-2 receptor, the T-lymphocytes will not proliferate. Azathioprine is a potent but non-specific immunosuppressant that inhibits both DNA and RNA synthesis by blocking *de novo* purine synthesis and causing breaks in the chromosomal DNA (Elion, 1975; Jensen, 1967; McCormack et al., 1982). Thus, cell proliferation is inhibited (Elion, 1975). The combination of corticosteroid and azathioprine was associated with a high incidence of acute rejection and infections (Costanzo, 1994). The introduction of cyclosporine to replace azathioprine decreased the acute rejection rate but also brought a high incidence of cyclosporine-induced acute and chronic nephropathy (Whiting, 1994). Because of this finding, the drug regimen was changed to include lower doses of oral corticosteroids, azathioprine and cyclosporine. The use of this triple therapy has resulted in lower rejection and infection rates and improved survival. Sometimes, long-term administration of corticosteroids will cause numerous severe adverse effects. To avoid these complications, some transplant centers withdraw recipients from corticosteroids beginning at variable post-operative intervals and using different tapering schedules. The use of this approach has been associated with a decrease in post-operative hyperlipidemia and hypertension. However, approximately one-third of the patients in whom corticosteroid withdrawal is attempted will have acute rejection and will ultimately require long-term corticosteroid therapy (Costanzo, 1994). Over the past five years, several new pharmacological agents have been shown to attenuate allograft rejection. These agents include macrolides (FK506 and rapamycin), guanidine (15-deoxyspergualin), mycophenolic acid (RS 61443), and prostaglandin analogs (Morris, 1996). There are also several biological agents such as soluble cytokine receptors and antibodies against surface antigens (eg. TcR/CD3 and ICAM-1) showing promise for immunosuppression. These types of therapy introduce short-term immunosuppressive treatment protocols and may lead to specific tolerance to the allograft.

## FK506

FK506 is a lipophilic macrolide lactone of 822 daltons (monohydrate) (Figure I-1) commonly used in post-transplantation therapy. It is a natural product from the antinomycete, *Streptomyces tsukubaensis*, (Hooks, 1994) and was discovered by scientists from Fujisawa Pharmaceutical Company, Ltd., Japan. The structure of FK506 was deduced early in the development through degradation and spectroscopy studies and X-ray crystal analysis. The definitive configuration was established by hydrolysis to L-pipecolic acid. Before the discovery of FK506, cyclosporine and other broad-spectrum drugs were the only sources of immunosuppressants. They all show extensive side effects because of the necessity of high dosages. The introduction of FK506 and several other new drugs such as rapamycin and mycophenolic acid led to decreased dosages for patients because of their increased potency compared with cyclosporine. FK506 itself was found to be approximately 100-fold more active than cyclosporine (McDiarmid et al., 1995; Zeevi et al., 1987). Therefore, the risk of complications because of toxicity may decrease for patients under the newer immunosuppressive therapy. Several comparisons of the side effects of cyclosporine and FK506 therapy have been made (McDiarmid et al., 1995; Porayko et al., 1995). In hepatic transplantation, the use of FK506 has been associated with improved patient and allograft survival, lower re-transplantation rates, and with the requirement for lower dosages of corticosteroids than those needed for cyclosporine-treated historical controls. As in renal transplantation, no clear advantage over cyclosporine was seen in the survival rates of grafts and patients (Shaw et al., 1996). However, treatment for hypertension decreased for patients receiving FK506. The incidence of hyperlipidemia or hypercholesterolemia for both hepatic and renal transplantation was similar in the two groups treated with FK506 or cyclosporine (Armitage et al., 1991). The most serious adverse effects associated with the use of FK506 are more extended with intravenous administration of the drug and include neurologic and renal toxicity as well as the onset of diabetes mellitus (McCauley et al., 1990; Mieles et al., 1990; Mieles et al., 1991; Shapiro et al., 1990; Starzl et al., 1989; Van Thiel et al., 1990).

## Mode of Action

FK506 is a drug with multiple mechanisms (Plosker et al., 2000; Dumont, 2000). In the immune system, FK506 affects T-cell function by inhibiting the production of IL-2, IL-3, IL-4, granulocyte stimulation factor (GSF), and interferon (INF) $\alpha$ (Tocci et al., 1989, Yoshimura et al., 1989). In order to mediate its effects, FK506 must bind with high affinity to its cytosolic target protein termed FK506-binding protein (FKBP), mainly FKBP-12. FK506 together with FKBP possesses immunosuppressive activity through its ability to interact with a serine threonine phosphatase, calcineurin (CN). Calcineurin, a heterodimer of calcineurin B-$Ca^{2+}$ binding subunit and calcineurin A-catalytic and calmodulin binding subunit, is a calmodulin- and $Ca^{2+}$-regulated protein phosphatase (Kincaid et al., 1991; Liu et al., 1991). Calcineurin dephosphorylates several substrates, including smooth and skeletal muscle myosin light chains, phosphatase inhibitor I, the $\alpha$ subunit of skeletal muscle phosphorylase kinase, and type II regulatory subunit of cAMP-dependent protein kinase (Blumenthal et al., 1986). Transcription factors (e.g. nuclear factor of activated T lymphocytes, NF-AT) that promote cytokine gene activation are either direct or indirect substrates of calcineurin. The binding of the FK506/FKBP complex to calcineurin reduces the enzymatic reaction between calcineurin and NF-AT. Thus, the transcription of cytokines responsible for lymphocyte activation is suppressed (Cliostone et al., 1993; Morris, 1996; Schreiber et al., 1992; Wiederrecht et al., 1993).

FKBP-12 and calcineurin are common constituents of lymphoid and non-lymphoid cells. FKBP-12 itself has been found to be associated with the ryanodine receptor and $IP_3$-associated $Ca^{2+}$ channels, and serves as a stabilizer of calcium release channels (Brillantes et al., 1994; Cameron et al., 1997). Intracellular calcium-release channels play crucial roles in $Ca^{2+}$-mediated signaling that triggers excitation-contraction coupling, T-lymphocyte activation, fertilization, and many other cellular functions. The cis-trans peptidyl-prolyl isomerase activity of FKBP-12 is believed to be required for optimizing channel function. FKBP-12 can also regulate cell proliferation via binding transforming growth factor (TGF)-$\beta$ receptor and cyclin-dependent kinase (CDK) (Khanna et al., 1999). Hence, the function of FKBP12 may be influenced when FK506 or its metabolites form a complex with FKBP12 (Cameron et al., 1997).

Several other FKBPs have been identified. FKBP51/FKBP52 are believed to be associated with the complex of heat shock protein 70/90 (Bruner et al., 1997). Transcription of pro-inflammatory cytokine genes may be inhibited by heat shock protein-mediated interaction between FK506 and the glucocorticoid receptor, which enhances translocation of the glucocorticoid receptor to the nucleus and potentiates binding to response elements (Plosker et al., 2000). FKBP52 is also found to play a significant role in the ability of FK506 to accelerate nerve regeneration in the rat peripheral nervous system (Gold, 1997; Gold, 2000).

## Pharmacokinetics

FK506 is highly lipophilic, with good solubility in methanol, chloroform, acetone, and propylene glycol (Honbo et al., 1987). Thus, the pharmacokinetics of FK506 is highly variable among individuals because of varying percentages of adipore tissue, and can be defined adequately by a two-compartment model (Venkatara et al., 1990). When FK506 is administered through a short period of intravenous infusion, it reaches peak concentration around two to four hours and then levels decline rapidly (Hooks, 1994). This indicates a rapid distribution of the drug from the central compartment. However, the decline of drug concentration becomes slower over the next 24 hours after reaching distribution equilibrium. As for the oral administration of FK506, the absorption is poor, erratic, and incomplete. The time to peak concentration varies from one to four hours. The absolute bioavailability for FK506 ranges from 6 to 56 percent, with the average around 22 (Aweeka et al., 1993; Mekki et al., 1993; Undre et al., 1999). This poor bioavailability may be caused by poor dissolution of FK506 in gastric fluids, efflux mediated by the P-glycoprotein pump, or by the first-pass metabolism occurring in the intestine and liver metabolic system (Shaw et al., 1999). Because the bioavailability is poor, three to four times higher doses may be required orally compared with intravenous doses (Hooks, 1994). The peak concentration of FK506 averages 45 μg/L after oral administration, with a corresponding mean time to peak concentration of 1.5 hours (Undre et al., 1999). The volume of distribution (Vd) for FK506 is approximately 1.5 L/Kg (Lee et al., 1993). Extra-vascular uptake of FK506 in lung, kidney, heart, and spleen has been demonstrated. This in part explains the apparently large Vd. In blood, FK506 is highly distributed into red blood cells (RBCs) and has a RBC-to-plasma ratio higher than 4:1. The partition of FK506 between RBCs and plasma depends on temperature, the higher the temperature the higher the partition in plasma (Hooks, 1994). Therefore, proper blood sample preparation is critical for analysis of FK506 in the plasma fraction. Within the plasma fraction, FK506 is found primarily in lipoprotein-deficient plasma (Warty et al., 1991). This is in direct contrast with cyclosporine, which is bound by various lipoprotein fractions. Alpha$_1$-acid glycoprotein is the most probable plasma protein involved in the transportation of FK506 in plasma (Hooks, 1994).

## Metabolism & Elimination

Drug metabolism is defined as the chemical modification of a drug in a biological environment. The procedure is also referred to as drug biotransformation. Most drugs undergo metabolic modification in the body to become more water-soluble metabolites which are then eliminated. Drugs that are metabolized may be converted to many products or may form only one major metabolite. Normally, drug metabolism is enzymatically controlled via oxidases, reductases, esterases, and enzymes involved in conjugation reactions. Sometimes, nonenzymatic reactions may occur in the drug metabolic reaction (eg. Cocain). Drug metabolic reactions can be classified as phase I or phase II reactions. Phase I metabolic reactions involve the introduction of a new chemical group into the drug molecule, especially by oxidative, reductive, or hydrolytic methods. Phase II reactions involve the conjugation of a small endogenous molecule to the parent drug or phase I metabolites. In general, the more water-soluble products of phase I reactions still require conjugation to be efficiently eliminated (Shen, 1997). The increased water solubility reduces tubular reabsorption in the kidney, resulting in excretion of these compounds in the urine (Baker et al., 1998).

Numerous examples of phase I metabolic reactions have been identified; many are oxidative reactions involving the introduction of oxygen into the molecule. This reaction is catalyzed by the mixed-function oxidases of the endoplasmic reticulum of the liver and other tissues. This system is referred to as the cytochrome P450 (CYP) family and requires molecular oxygen and NADPH (or NADH) as co-factors for the reactions. Cytochromes P450 are heme-containing proteins that are found in endoplasmic reticulum and mitochrondria. The CYP enzymes are important in the metabolism of various endogenous compounds such as bile acids, fatty acids, leukotrienes, prostaglandins, steroids, biogenic amines, retinoids, lipid hydroperoides, and phytoalexins. These enzymes also metabolize drugs, environmental chemicals and pollutants as well as natural plant products involved in flavor, odor, flower color and the response to inflammation (Nelson et al., 1996). Of all 74 gene families that have been identified, at least 14 different gene families (1, 2, 3, 4, 5, 7, 8, 11, 17, 19, 21, 24, 27, and 51) of CYP enzymes exist in humans (Nelson et al., 1996). CYPs 4, 5, 7, 8, 24, 27, and 51 are involved in biosynthesis and metabolism of substances such as bile acids, fatty acids, eicosanoids, and vitamins, while CYPs 11, 17, 19, and 21 are involved in the biosynthesis of steroid hormones from cholesterol. Families 1, 2, and 3 are implicated in the metabolism of various drugs and xenobiotics. Some of the gene families contain subfamilies, each with different capital letters. Members of the same subfamily, each distinguished by a terminal Arabic number, have greater than 55% amino acid sequence similarity (Baker et al., 1998). The enzymes that have garnered the most interest in drug metabolism are CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP1A2 and CYP2E1 (Meyer, 1996; Bertz et al., 1998). Approximately 30% of the drugs currently in clinical use are known to undergo metabolism by CYP2D6 (Lewis et al., 1997). Characteristics of this enzyme include an acidic binding site for the basic nitrogen of the substrate (Smith, 1991). The substrates tend to be lipophilic and strong organic bases that become protonated at physiological pH. CYP2E1 is responsible for metabolizing approximately 1% of drugs (Lewis et al., 1997). This enzyme has been studied in relationship to the formation of toxic metabolites and alcoholism (Bertz et al., 1997). Expression of CYP2C19 has not been detected in extrahepatic tissue (Gonzalez, 1992). Enzymes of the CYP2C subfamily account for the metabolism of about 15% of all known drugs that undergo metabolism (Lewis et al., 1997). The significance of CYP3A4 will be discussed later.

Most examples of phase II metabolism involve compounds whose structure includes R-OH. This type of compounds can be metabolized to R-XH forms, X= NH, NR', O or S. Of all the phase II metabolic reactions, conjugation with glucuronic acid is the most important in humans (Jakoby, 1980.). Glucuronidation requires the cofactor uridine diphosphate-glucuronic acid (UDP-glucuronic acid). The reaction is catalyzed by UDP-glucuronosyltransferases which are located in the endoplamic reticulum of liver and other tissues such as brain, kidney, intestine, skin, spleen, and nasal mucosa (Parkinson, 1996). Glucuronidation generally detoxifies xenobiotics and potentially toxic endobiotics. However, steroid hormones glucuronidated on the D-ring can cause cholestasis (Parkinson, 1996); and the induction of UDP-glucuronosyltransferase activity has been implicated as an epigenetic mechanism of thyroid tumor formation in rodents (Curran et al., 1991; McClain, 1989).

FK506 is almost completely metabolized by enzymes of the CYP 3A subfamily prior to elimination. The major phase I mechanisms involve monodemethylation, didemethylation, hydroxylation, or combinations of the above (Hooks, 1994). There is very little known about phase II metabolism of FK506. CYP 3A4 is the major iso-enzyme involved in the phase I metabolism of FK506 (Sattler et al., 1992). CYP 3A4 is involved in the metabolism of more than 50% of the drugs used in humans, and it accounts for 30% of total CYP based on hepatic protein mass (Colbourne, 1999). CYP3A4 is very similar to CYP3A3 with only an 11 amino acid difference and no known catalytic differences. CYP3A4 appears to be the most abundant of these two forms in liver (Gonzalez, 1992). CYP3A4 can be inhibited by itraconazole, ketoconazole, and troleandomycin; and can be activated by phenobarbital, carbamazepine, dexamethasone, phenytoin, rifampin, and sulfadimidine (Parkinson, 1996). Thus, drugs that enhance CYP3A4 may lead to subtherapeutic levels of FK506 whereas drugs that inhibit the CYP3A4 may lead to increased levels of FK506 and the potential for drug toxicity (Green et al., 1999). To date, several drugs have been found to interact with FK506 and result in an increase or decrease of FK506 concentrations in trough human blood. Concomitant immunodeficiency virus protease inhibitors, e.g. saquinavir, ritonavir, and nelfinavir, can increase FK506 blood levels (Schvarcz et al., 2000; Sheikh et al., 1999). Co-administration of antifungal agents, ketoconazole, itraconazole, and fluconazole, can also increase FK506 concentrations in blood (Venkatakrishnan et al., 2000; Outeda et al., 2000; Capone et al., 1999). A calcium blocker, mibefradil, increases the blood concentrations of FK506 and leads to severe FK506-related toxicity (Ocran et al., 1999). Clarithromycin and diltiazem have also been shown to increase blood concentrations of FK506 and thus side effects (Gomez et al., 1999; Hebert et al., 1999). On the other hand, rifampin can decrease FK506 concentrations in blood, thus requiring an increase in the dose of FK506 necessary to prevent graft rejection (Chenhsu et al., 2000).

After FK506 is metabolized in the small intestine (Lampen et al., 1995) and the liver, most of the metabolites are excreted in the bile then feces. Urinary excretion accounts for less than 3% of total administered dose (Möller et al. 1999). The total body clearance of FK506 is approximately 4.1 L/h in liver transplant patients and 6.8 L/h in renal transplant patients (Lee et al., 1993; Mekki et al., 1993). This clearance appears to be greater than that of cyclosporine, although this may depend on the analytical method of the study and whether whole blood or plasma was used. The half-life for FK506 in patients with normal liver function is approximately 8.7 hours, and is prolonged in patients with hepatic dysfunction (Venkataramanan et al., 1991). During episodes of impaired liver function in patients, the concentrations of FK506 and its metabolites are increased compared with the situation in patients having normal liver function. This indicates accumulation of FK506 metabolites (Venkataramanan et al., 1991).

Seven FK506 metabolites have been structurally identified using rat liver microsomal preparations (Iwasaki et al., 1993; Iwasaki et al., 1995) (Figure I-1), and nine metabolites have been identified from human blood, urine, and bile

(Christians et al., 1991; Christians et al., 1992) prior to my study. These studies have demonstrated that 31-demethyl FK506 exhibited immunosuppressive activity which was comparable to that of the parent drug, while 31-demethyl FK506, 15-demethyl FK506, and 15,31-didemethyl FK506 exhibit significant cross-reactivity with the antibody in both immunoassays (Iwasaki et al., 1995; Lhoëst et al., 1994). The rest of the identified metabolites did not contribute significantly to the immunosuppressive activity or cross-reactivity.

## Side Effects

All immunosuppressants have some associated side effects, with infection and malignancy being common among them. Patients lose their ability to defend themselves from microorganisms and cancer cells because of the inhibition of the immune system (Costanzo, 1994).

The infections are viral (15%), bacterial (34%), fungal (9%) and protozoan (8%). Opportunistic infection can be transmitted by the donor and blood products or may be caused by the reactivity of air, water, or fecal organisms (Gottesdiener, 1989). Infections will remain a frequent complication after transplantation and can be masked if patients are also receiving corticosteroid therapy. Therefore, even mild elevations of the patient's temperature should not be disregarded and the etiology should be aggressively investigated. A thorough history and physical examination, including complete blood count with differential lymphocyte typing, chest x-ray and bacterial, viral and fungal cultures for blood, urine, and sputum may reveal the localized signs. Sometimes, an open lung biopsy may be necessary for definitive diagnosis. Patients with fever and gastrointestinal symptoms should be examined by endoscopy with brushing and biopsy for culture and staining to identify cytomegalovirus (CMV) inclusion bodies. Computerized axial topography and lumbar puncture should be done on patients with neurological abnormalities.

Post-transplant malignancy is a well-recognized complication of immunosuppression (Penn, 1978). Skin cancer is the most common cancer occurring in transplant patients. Lymphoproliferative disorder represents a spectrum of abnormal B cell proliferation that occurs with variable frequency for patients under immunosuppression. The risk of post-transplant lymphoproliferative disorder appears to be associated with the degree of total immunosuppression. Epstein Barr Virus (EBV) infection often precedes the appearance of post-transplant lymphoproliferative disorder and may result from inadequate T cell control over EBV-driven B cell proliferation. Lymphoma should be suspected in transplant recipients who show fever of undetermined etiology, systemic symptoms, and elevated EBV titers. Some of the lymphoproliferative disorders, particularly when occurring early after transplantation, may have a fulminating clinical course with widespread tumors, organ failure, and systemic sepsis (Penn, 1983). Aggressive evaluation should include assessment of the gastrointestinal tract and central nervous system. Tissues should be obtained from any suspicious lesions for histologic and lymphocyte phenotyping. Bone marrow aspiration and biopsy should be done to rule out the involvement of lymphocyte phenotyping differences.

The side effects of FK506 in transplantation therapy include nephrotoxicity (Whiting, 1994; de Mattos et al., 2000), hypertension, neurotoxicity (Wijdicks et al., 1994), and abnormal glucose metabolism (Yanchar et al., 1996; Weir et al., 1999). Many of these adverse effects, such as nephrotoxicity, neurotoxicity, glucose disturbances and infections are, at least in part, dose-related and can often be managed by dose reduction (Plosker et al., 2000).

Nephrotoxicity is the major side effect from FK506 immunosuppressive therapy. In the major trials for patients undergoing liver or kidney transplants, nephrotoxicity occurred in as many as half of patients treated with either FK506 or cyclosporine (Mayer et al., 1997; Pirsch et al., 1997). Increased serum creatinine was the most frequently reported indicator of renal toxicity, but oliguria and kidney failure were also reported in some patients (Plosker et al., 2000). Scientists have demonstrated that the functional and morphologic alterations of kidneys caused by FK506 are similar to those caused by cyclosporine in a rat model (Stillman et al., 1995; Shaw et al., 1996; Mollison et al., 1998). There are two types of nephrotoxicity, acute and chronic, that are caused by FK506 (McCauley, 1993; Nielsen et al., 1995; Andoh et al., 1995; Porayko et al., 1994). Acute nephrotoxicity is reversible and appears to be related to the dosage of FK506. It is caused by a reduction in renal blood flow related to afferent arteriolar vasoconstriction. Chronic nephrotoxicity is an irreversible interstitial fibrosis that develops after approximately 6 to 12 months of drug therapy. Chronic nephrotoxicity is the major limitation on the use of FK506 because patients who develop this side effect can often progress to end-stage renal disease (de Mattos et al., 2000). Effective strategies for reducing the risk of acute and chronic nephrotoxicity are dosage adjustment to maintain blood concentrations of the parent drug within a narrow target range and concomitant therapy with calcium channel-blocking anti-hypertension agents (Whiting, 1994). This may attenuate some of the FK506-mediated renal hemodynamic changes but may not affect the more chronic histological changes. Sometimes, patients on a low-dose FK506-based regimen still develop nephrotoxicity. In a comparison with patients on a cyclosporine A-based regimen, nephrotoxicity occurred in 20% of patients receiving a low dose of FK506 versus 14.3% of patients on cyclosporine A (Kokado et al., 1998). The condition of the kidney itself also contributes to the nephrotoxicity. Thus, improving the surgery and preservation techniques for the organs can reduce the possibility of kidney dysfunction after the transplantation. Introducing the new types of therapy with fewer overt side effects may eliminate nephrotoxicity, although new types of side effects may be introduced (Hooks, 1992). In fact, there is very little known about how FK506 causes its nephrotoxic side effects.

Neurotoxicity associated with FK506 most frequently displays as tremor, headache, insomnia and paraesthesia, and some neurological effects are more problematic with FK506 than with cyclosporine (Mayer et al., 1997; Pirsch et al., 1997). Tremor occurred in 35 to 56% of FK506 recipients in the major clinical trials in kidney and liver transplantation, and was significantly more common in FK506 than cyclosporine recipients (Plosker et al., 2000). Severe neurotoxic effects such as seizures, akinetic mutism, expressive aphasia, coma, and delirium have also been reported rarely, and are associated with high whole blood concentrations of FK506 (Mor et al., 1997; Prograf prescribing information, 1998). Liver transplant recipients, especially those with hepatitis B or C, may be at particular risk of severe FK506-induced neurotoxicity (Mueller et al., 1994; Chang, 1999).

Post-transplantation diabetes mellitus and/or hyperglycemia tended to occur more frequently with FK506 than with cyclosporine in the major trials in kidney or liver transplant recipients (Mayer et al., 1997; Pirsch et al., 1997). The incidence of new-onset type one diabetes mellitus was as much as five times greater with FK506 than cyclosporine in renal trials, 20 vs. 4% in the US trial (Pirsch et al., 1997) and 8 vs. 2% in the European study (Mayer et al., 1997). The incidence for all diabetes mellitus in the European trial was 12% with FK506 versus 2% with cyclosporine (Mayer et al., 1997). In a more recent study of 208 renal transplant recipients receiving FK506-based immunosuppression, the initial and final incidences of post-transplantation diabetes mellitus (mean follow-up of 15 months) were 7.0% and 2.9% respectively (Shapiro et al., 1999). The lower incidence of post-transplantation diabetes mellitus in this recent trial relative to previous reports suggests that it may be possible to reduce the risk of developing this side effect by FK506 dosage reduction or by the combination of various drugs in the immunosuppressive therapy (Shipiro et al., 1999; Shipiro et al., 1999 letter). Filler et al. (2000) have shown that FK506 can reversibly reduce insulin secretion in pediatric renal transplant recipients. Risk factors that have been identified for the development of post-transplantation diabetes mellitus include ethnic race (African-American or Hispanic, 3.3 fold increased risk), high trough blood concentration of FK506 and high corticosteroid dosage (Pirsch et al., 1997). Fasting glucose levels should be monitored regularly during FK506 therapy (Plosker et al., 2000). Little is known about the mechanism of post-transplantation diabetes mellitus caused by FK506, although a small number of studies have been conducted in an effort to understand the mystery of the mechanism. Normal insulin sensitivity (post-hepatic insulin effect) and enhanced second-phase insulin secretion (pre-hepatic insulin effect) in FK506 treated liver transplant patients have pointed to an accelerated hepatic insulin clearance rate (Konrad et al., 2000). Increased hepatic insulin clearance can be compensated for by enhanced insulin secretion, indicating that insulin clearance is the major determinant of pancreatic function in liver-grafted patients (Konrad et al., 2000). The alterations in glucose tolerance and insulin secretion observed with the use of FK506 have been associated with morphological changes to the islet cells, including significant vacuolization and degranulation that frequently resolved with discontinuation of the drug (Drachenberg et al., 1999; Weir et al., 1999).

## Therapeutic Drug Monitoring of FK506

There are four main reasons why drug levels need to be monitored during therapy: (a) The therapeutic window is very narrow (toxic symptoms occur at levels close to those giving the therapeutic effect). (b) Large intra- and inter-individual variabilities exist in the pharmacokinetics of the drugs. (c) Other factors such as co-administered drugs can interact with the pharmacokinetics or pharmacodynamics of the drugs of interest. (d) Not all patients reach the desired therapeutic effects at standard doses (Lindholm et al., 1995). All of these points apply to FK506, thus necessitating therapeutic drug monitoring of FK506. Designing a sensitive and specific analytical method to measure concentrations of FK506 for therapeutic monitoring becomes an important part of optimizing therapy for FK506. Several analytical methods for the measurement of FK506 in biological specimens have been developed, and these assays have been used for therapeutic drug monitoring.

Whole blood is the preferred matrix for therapeutic blood monitoring of FK506 as the drug is extensively bound to red blood cells (Jusko et al., 1991; Jusko, 1995). However, plasma FK506 is closer to the "free" state of the drug. Most drugs act when not bound to plasma proteins or red blood cells (Jusko, 1995). Some researchers have suggested that both whole blood and plasma should be monitored in order to estimate the effects of FK506 (Jusko, 1995). FK506 is stable in blood one week at room temperature, and for six months or more at – 20 °C (Jusko, 1995).

Two assays are commercially available for monitoring FK506 in whole blood. Both of them are immunoassays utilizing the same monoclonal antibody to FK506. The IMx assay (Abbott Laboratories, Abbott Park, IL, USA) is based on microparticle enzyme immunoassay (MEIA) technology, while the Pro-Trac assay (INCSTAR Corporation, Stillwater, Minnesota, USA) utilizes the enzyme linked sorbent immunoassay (ELISA) technique. The detection limit for the Pro-Trac technique (0.3 μg/L) is lower than that of IMx assay (2 μg/L) (Alak, 1997).

Two key problems with clinical FK506 monitoring are the establishment of appropriate post-transplant time-dependent consensus therapeutic ranges, and the influence of possible cross-reactivity of FK506 metabolites with anti-FK506 used in the immunoassays, especially for patients with liver dysfunction. A target range of 5-15 μg/L for trough whole blood concentration has been recommended and used clinically (Oellerich et al., 1998; MacFarlane et al., 1999). The incidence of toxicity is about 45% for patients showing trough blood concentrations above 15 μg/L. The time-dependent therapeutic range of FK506 trough blood concentrations differs among transplant patients (Oellerich et al., 1998; MacFarlane et al., 1999), but most use the higher range of target concentrations during the early postoperative period. The dosage is then tapered to a lower maintenance concentration range. There is evidence that FK506 metabolites can cross-react with the anti-FK506 used in commercial immunoassays (Alak, 1997). This will result in an over-estimation of the FK506 concentrations and lead to under-dosing of the patients and an increased incidence of graft rejection. If this happens in patients with liver dysfunction or cholestasis, the influence of over-estimation will increase because of the accumulation of the metabolites or the long half-life of them in the lipoid environment. The adjustment of the dosage may not truly reflect the concentration needed for patients.

Liquid chromotography/mass spectrometry (LC/MS) and liquid chromotography/mass spectrometry/mass spectrometry (LC/MS/MS) are also used to identify FK506 metabolites in biological fluids (Taylor et al., 1996; Zhang et al., 1997). Traditionally, LC is used with UV spectrometry to measure chemicals. FK506 and its metabolites have similar maximal absorption values at a wavelength of 214 nm under UV light as the solvent used in the analysis system. The concentrations of FK506 and its metabolites in biologic fluids are also very low (less then 20 μg/L). These two reasons make it difficult to detect FK506 and its metabolites using UV spectrometry. Thus, a more sensitive method such as LC/MS or LC/MS/MS is used as a reference method.

### Liquid Chromatography/ Mass Spectrometry (LC/ MS)

Chromatography is a collective term applied to a wide variety of separation techniques based upon the sample partitioning between a moving phase that can be gas or liquid and a stationary phase that can be either a liquid or a solid (Johnson et al., 1978). The two principal classes of chromatographic methods are gas chromatography and liquid chromatography classified according to the nature of their mobile phase. The discussion that follows will focus on high performance liquid chromatography (HPLC) and on one of the most frequently used detection systems for FK506 monitoring-- mass spectrometry (MS).

HPLC is a technique used to separate the components of a chemical mixture. These components are first dissolved in a liquid solvent, and then forced to flow through a chromatographic column under high pressure. In this column, the mixture is resolved into its components. The amount of resolution is dependent upon the extent of interaction between the solute components and the stationary phase. The moving part of the system is the mobile phase, which is a liquid. The stationary phase is coated on the immobile packing material in the column. The interaction of the chemical components with mobile and stationary phases can be manipulated through different choices of solvents and columns. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems. Thus, HPLC has the ability to easily separate a wide variety of chemical mixtures (Schram, 1981). Liquid chromatography can be subdivided in four types: liquid/ liquid chromatography (LLC), liquid/solid chromatography (LSC), ion- exchange chromatography, and exclusion chromatography. LSC is an adsorption chromatography that uses adsorbents such as silica gel, alumina, molecular sieve or porous glass packed in a column. Reverse-phase liquid chromatography, a type of LLC, was used for the experiments in my study. The columns of this type are usually preferred to aryl-/alkyl-silane-bonded (eg. $C_{18}$) (LeGatt, 1988). A liquid chromatography system usually consists of a reservoir for mobile liquid, pump, injection port (sample inlet), column, detector, recorder, and thermostats for the column and detector (Hadden et al., 1971). The retention or elution time is the time from the point of injection to the peak maximum of one particular component. It is a characteristic property of a given compound on a specific column under a given set of conditions such as temperature, pressure, liquid phase, mobile phase, and flow rate of the mobile phase. When experimental conditions are controlled, the retention time of a given compound is reproducible and can be used to help identify samples. The area of each peak in a chromatogram is proportional to the concentration of the peak's component. It is possible to determine the exact concentrations of the components if a standard curve is established (Hadden et al., 1971).

The combination of high performance liquid chromatography (HPLC) and mass spectrometry (MS) in a tandem technique is extremely attractive for the characterization of polar and labile compounds, especially where complex mixture analysis is required. MS is a technique where ions, depending on their mass-to-charge ratio (m/z), can be separated and detected either universally or selectively depending on the scan mode. In a mass spectrometer, five different events are performed: sample introduction, analyte ionization, ion separation, ion detection, and data handling. The sample is introduced through an interface into the ion source and charged species are created from the analytes of interest utilizing different kinds of ionization techniques (Sjöberg, 1999). When a molecule goes into the mass spectrometer from the sample inlet, it will be ionized either by electrons directly or by electrons from an inert chemical such as helium. A large number of ionization methods have been used in the past, and each one has its own merits and limitations. Traditional methods such as electron impact ionization tend to transfer a large amount of energy into the ionized molecule which usually gives rise to extensive fragmentation. Fragmentation information can be used for molecular structure interpretation (McLafferty et al., 1993). Several more recently developed ionization techniques can produce less fragmentation and these methods are often referred to as "soft" or "mild" ionization methods. These "soft" ionization methods have the potential to form ions from fragile molecules, usually as protonated or deprotonated species which provide molecular weight information (Sjöberg, 1999). One of the soft ionization methods, namely electrospray ionization, was used in my experiments. It is a technique where a liquid is dispersed into small charged droplets by an electrostatic field (Sjöberg, 1999). The droplets are formed at the end of a capillary, and when the droplet size increases, the surface tension can no longer counteract the gravitational force. Thus, the droplet will fall down. If an electric field is generated between the capillary tip and a counter electrode, ions present in solution will migrate under the influence of the field to the opposite direction of the field until a charge distribution is obtained to counteract the field. The electrical field-induced ion evaporation can generate gas phase ions at atmospheric pressure from charged droplets and these ions can be detected with mass spectrometry (Sjöberg, 1999). The ion then passes through the repeller and the ion focuser and is accelerated. The accelerated ion passes through the mass filter and is captured by the detector, the mass spectrum is generated by altering the electrical field of mass filter over time. Only one particular mass/charge (m/z) ion will be detected at a given time. A quadrupole analyzer, the most commonly used analyzer today, was used to detect the ion mass in my experiments. A quadrupole analyzer consists of four parallel metal rods which are arranged symmetrically. By applying radio frequency (RF) and direct current (DC) potentials to the rods, a quadrupole electric field is created that can filter ions according to their m/z ratio (Dawson, 1976; Sjöberg, 1999). The abundance vs. m/z ratios are then plotted. The results from LC/MS can be presented in both qualitative and quantitative ways. When experimental conditions are controlled, the retention time and m/z ratio of a given compound are reproducible and can be used to identify the sample. The area of each peak in a spectrum is proportional to the concentration of the peak's component, and thus the mass spectrometer can be used to quantify the compound of interest if a standard curve is established.

## Problems with FK506 Immunosuppressive Therapy

1. There is currently a lack of knowledge about FK506 metabolites:

Although it has been confirmed that demethyl-FK506 is the major metabolite, followed by demethyl-hydroxy-FK506, in human blood (Gonschior et al., 1996), the concentrations of the same species of the metabolite (e.g. demethylated FK506) with positional differences have not been compared. The minor FK506 metabolites have yet to be studied extensively. The distribution of

FK506 metabolites in different tissue types also has not been evaluated. The possibility of formation of phase II metabolites has not been investigated. Each metabolite may have different biological functions, i.e. some of them may contribute to the total immunosuppression and some to the toxicity, but very little is known about the overall biological activity of each metabolite.

2. The steady state concentrations of the metabolites of FK506 in human blood are unknown:

A knowledge of the concentrations of FK506 and its metabolites in the body fluids is required in order to understand the role of metabolites in FK506 immunosuppression and toxicity. In order to investigate the steady state concentration of each metabolite in human whole blood, a suitably sensitive method, e. g. LC/ MS, needs to be established.

3. The reliability of immunoassays used clinically to monitor FK506 has not been fully studied:

In order to assess the specificity of MEIA and ELISA methods, comparative studies with LC/MS should be conducted. Generally speaking, a positive bias would be expected with MEIA and ELISA compared with LC/MS. The positive bias is presumably due to the cross-reactivity of several FK506 metabolites with the monoclonal antibody used in the immunoassays.

4. There is a lack of knowledge about the mechanisms of FK506-related toxicity:

The major side effects of FK506 immunosuppressive therapy are renal toxicity, hypertension, neurotoxicity, and abnormal glucose metabolism (Whiting, 1994; Wijdicks et al., 1994; Yanchar et al., 1996; de Mattos et al., 2000; Weir et al., 1999). Damage to the vasoconstriction-vasodilatation regulatory system of renal vaso-endothelium may be the main reason for the renal toxicity (Copeland et al., 1992; Langman et al., 1994; Goodall et [illegible] 1995). The mediators in this system include vasodilator substances such as prostacyclin (prostaglandin $I_2$, $PGI_2$) and nitric oxide (NO), and vasoconstrictor autocoids, the most potent of which is the 21-residue peptide, endothelin-1 (ET-1) (Langman et al., 1994; Goodall et al., 1995). $PGI_2$ and NO inhibit, whereas ET-1 enhances the proliferation of endothelium cells, smooth muscle cells, and mesangial cells. Angiotensin II, bradykinin, IL-1, and IL-6 are some of the factors that control the upstream regulation of the vasoconstriction-vasodilatation system (Agui et al., 1994; Chen et al., 1994; D'Souza et al., 1994; Vajo et al., 1996; Bustos et al., 1997; Odoux et al., 1997; Yokoo et al., 1997). The relationships between these factors need to be investigated.

Recently, some evidence that the toxic effects of FK506 may be mediated through binding to FKBP-12 and other FKBPs in nonimmune cells was reported (Cameron et al., 1997). Thus the relationship between FK506 (and its metabolites) and the FKBPs and the subsequent influence on the micro-mechanisms of cell growth and maintenance need to be investigated.

## Objectives and Rationale

Therapeutic drug monitoring of FK506 has been an integral part of transplantation in order to optimize the effects of immunosuppression while minimizing toxicity of the drug.

In previous studies of the pharmacokinetics and metabolism of FK506, the assumption was made that the role of FK506 metabolites in monitoring and determining the biological functions of FK506 can be ignored (Alak, 1997). This assumption should be challenged until the overall immunosuppression, toxicity, and steady state concentrations of FK506 metabolites are fully studied. If the contributions of FK506 metabolites to the biological function and toxicity of the drug are sufficiently low, it is then hypothesized that the therapeutic monitoring of FK506 can rely solely on the concentration of only the parent drug in whole blood. The overall hypothesis of my study was based on the statements above: FK506 metabolites may play significant roles on the biological functions and toxicity of FK506. Thus, the objectives of this study were the following:

1) To establish an LC/MS assay as a reference method to identify and monitor FK506 metabolites in biological fluids.
2) To establish an *in vitro* metabolism system in order to study the patterns of FK506 metabolites in different biological sources and

produce large quantities of FK506 metabolites for further purification and study.

3) To assess the reliability of immunoassays used clinically to monitor FK506 trough blood concentrations.
4) To determine the steady state concentrations of FK506 and its metabolites in whole blood.
5) To assess the biological functions of FK506 metabolites through the mixed-lymphocyte reaction and calcineurin inhibition.
6) To assess the degree of cross-reactivity of FK506 metabolites in FK506 immunoassays.
7) To assess the *in vitro* toxicity of FK506 and its metabolites on primary aorta endothelial cells and determine if the hypothesis that FK506 induces renal toxicity by influencing the release of vasodilators and vasoconstrictors from endothelial cells is true.

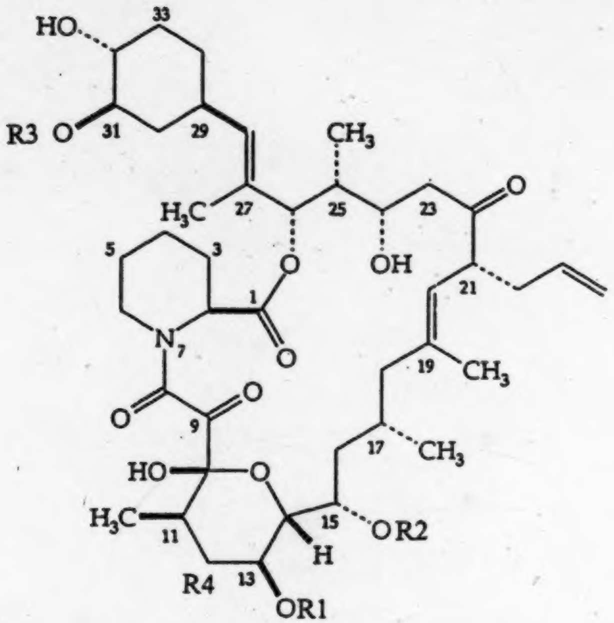

HO
33
R3 O
31
29
CH3
O
H3C
27
25
23
5
3
O
OH
21
1
N
7
O
19
CH3
O
O
9
17
CH3
HO
O
H3C
11
15
OR2
H
R4
13
OR1

Figure I-1: Chemical structure of FK506 and its metabolites.

| | R1 | R2 | R3 | R4 |
|---|---|---|---|---|
| FK506 | $CH_3$ | $CH_3$ | $CH_3$ | H |
| 13-demethyl | H | $CH_3$ | $CH_3$ | H |
| 15-demethyl | $CH_3$ | H | $CH_3$ | H |
| 31-demethyl | $CH_3$ | $CH_3$ | H | H |
| 13,15-didemethyl | H | H | $CH_3$ | H |
| 13,31-didemethyl | H | $CH_3$ | H | H |
| 15,31-didemethyl | $CH_3$ | H | H | H |
| 12-hydroxy | H | H | H | OH |

# Chapter II

## Identification and purification of FK506 metabolites

### Objective

In order to study the biological significance of FK506 metabolites, their formation from different biological sources was examined and the best system to produce large quantities of FK506 metabolites was chosen for the purpose of purification and further investigation.

### Materials and Methods

Purification of FK506 from pharmaceutical capsules

Materials

Methanol- Sigma-Aldrich, Canada. HPLC grade

Acetonitrile- Sigma-Aldrich, Canada. HPLC grade

Acetic acid- Caledon Inc. Canada.

Bench top centrifuge- IEC Centra GP8, Fisher, Canada.

Liquid chromatographic/Mass spectrometry (LC/MS) system- Series 1100, Hewlett Packard, Canada.

High Performance Liquid chromatography (HPLC) system- Series 1100, Hewlett Packard, Canada.

FK506 capsules (10 mg)- Fujisawa Co. USA

FK506 standard- Fujisawa Co. USA

$C_{18}$ High performance liquid chromatography (HPLC) column- Nova-Pak, 3.9 ×150 mm, Waters, USA.

Splitter- Acurate LC Packings, Canada.

Guard column- In-house prepared with $C_8$, Upchurch Scientific, inside.

Freeze Dry system- Freezone 4.5, Labconco Inc. Canada.

Benzene- Sigma-Aldrich, Canada.

Flow-injector- Harvard Apparatus 22, Fisher, Canada.

Nitrogen tank- Praxair, Canada.

pH meter- Accumet Basic, Fisher Scientific, Canada

Balance- AG204, Mettler- Toledo Inc. Canada.

Micro-balance- AE200, Mettler-Toledo Inc. Canada.

## Method

In order to obtain a large quantity of FK506 for subsequent experiments, FK506 was extracted from commercially available FK506 capsules. The powder inside the capsules that contained at least 10 mg FK506 was suspended in 10 mL of methanol by vortexing. The solid residues were removed by centrifugation, and the organic extracts were pooled. Some of the organic phase was first injected into the HPLC system and components in this phase were separated by using a methanol (MeOH)/acetonitrile (ACN)/acetic water (pH 3) gradient at a flow rate of 1.25 mL/min. pH 3 acetic water was made by dissolving acetic acid in deionized water to give a final concentration of 0.2% (v/v). The gradient profile was as follows: 0-15 minutes: 27% MeOH/30% ACN/43% pH 3 water → at 25 minutes: 40% MeOH/30% ACN/30% pH 3 water → at 30 minutes: 60% MeOH/30% ACN/10% pH 3 water → 35 minutes: 100% MeOH. FK506 was identified according to the mass of FK506 measured by the electrospray mass spectrometer and the elution time of a known FK506 standard. The FK506 standard was donated by Fujisawa Co. USA. The rest of the organic phase was injected into an HPLC system consisting of two C18 columns joined in tandem with a guard column before them. FK506 fractions were collected and taken to dryness under a stream of nitrogen. The residues were dissolved in benzene and taken to dryness in a freeze-dry system. The weight of the white, dry, pure FK506 powder was measured and stored at a concentration of 10 μg/mL in methanol. 1 μL of this FK506 stock was flow-injected by flow-injector at a rate of 50 μL/min through the MS system to confirm the purity of the drug.

### Preparation of acetyl-FK506 as an internal standard.

#### Materials

Acetonitrile- Sigma- Aldrich, Canada. HPLC grade

Acetic anhydride- Sigma- Aldrich, Canada.

FK506 stock- 10 μg/mL in methanol, from the capsule preparation

Nitrogen tank- Praxair, Canada.

Liquid chromatographic/Mass spectrometry (LC/MS) system- Series 1100, Hewlett Packard, Canada.

$C_{18}$ High performance liquid chromatography (HPLC) column- Nova-Pak,

3.9 ×150 mm, Waters, USA.

Splitter- Acurate LC Packings, Canada

Flow-injector- Harvard Apparatus 22, Fisher, Canada.

Methanol- Sigma- Aldrich, Canada. HPLC grade

Water bath shaker- VWR Scientific, Canada.

Balance- AG204, Mettler- Toledo Inc. Canada.

Microbalance- AE200, Mettler- Toledo Inc. Canada.

Method

FK506 (5 μg) in 5 mL of acetonitrile/water (70/30, v/v) was incubated with 5 mL of acetic anhydride ($C_4H_6O_3$) in a 75 °C water bath for 2 hours (Christians, 1992). Unused acetic anhydride was evaporated at 60 °C under a stream of nitrogen. The residue was dissolved in 1 mL of acetonitrile/water (75/25, v/v), pH=3. 1 μl of the solution was injected into a LC/MS system to identify FK506 and acetyl-FK506 peaks according to their masses. The rest of the solution was injected into a HPLC system that was the same as used for the purification of FK506. The acetyl derivative (peak number 2) was collected and taken to dryness under a stream of nitrogen and stored in the methanol as 10 μg/ml stock. The purity of this acetyl-FK506 was confirmed by flow-injectiion at a rate of 50 μL/min through the MS system.

## Development of LC/MS method to monitor FK506 and possible metabolites

### Materials

Methanol- Sigma- Aldrich, Canada. HPLC grade

Acetonitrile- Sigma- Aldrich, Canada. HPLC grade

Acetic acid- Caledon Inc. Canada.

FK506 stock- 10 μg/mL in methanol, from the capsule preparation

Nitrogen tank- Praxair, Canada.

Liquid chromatographic/Mass spectrometry (LC/MS) system- Series 1100, Hewlett Packard, Canada.

High Performance Liquid chromatography (HPLC) system- Series 1100, Hewlett Packard, Canada.

$C_{18}$ High performance liquid chromatography (HPLC) column- Nova-Pak, 3.9 × 150 mm, Waters, USA.

Drug- free human blood, from volunteers in our laboratory.

pH meter- Accumet Basic, Fisher Scientific, Canada.

### Method

A particular LC/MS system was developed as a reference method to monitor FK506 and possible metabolites. Two tandem $C_{18}$ HPLC columns heated at 50°C were used for HPLC separation. The target solution (FK506 standard or test solution) was first injected into the HPLC system and separated by using a methanol (MeOH)/acetonitrile (ACN)/acetic water (pH 3) gradient at a flow rate of 1.25 mL/min. The gradient profile was as follows: 0-15 minutes: 27% MeOH/30% ACN/43% pH 3 water → at 25 minutes: 40% MeOH/30% ACN/30% pH 3 water → at 30 minutes: 60% MeOH/30% ACN/10% pH 3 water → 35 minutes: 100% MeOH. The HPLC column effluent was then introduced into the mass spectrometer via an electrospray interface. The mass spectrometer was tuned to adjust the ion source, mass filter, and detector. The commercially available electrospray-tuning mix (HP G2421A) was used for the tuning. This mixture contains seven molecules of different molecular weights (118.09, 322.05, 622.03, 922.01, 1521.97, 2121.93, and 2721.89). The instrument was operated in selected ion monitoring mode. The masses of the ions were chosen depending on if FK506 alone or together with metabolites needed to be monitored. The molecular ion of FK506 was 826 with sodium add-up. The other metabolites were calculated according to their possible metabolism pathway such as demethylation or hydroxylation. When identifying FK506 metabolites, only the correct masses that did not appear in the LC/MS profile of a control drug-free blank extract were recorded. The recovery and accuracy of this LC/MS method were determined as follows: Pure FK506 was spiked into a drug-free blood pool to generate 10 μg/L and 25 μg/L standards. These samples were taken through the extraction procedure (details in next section) and analyzed by LC/MS. The recovery (extraction efficiency) for both FK506 and the internal standard was determined by comparing peak areas from extracted samples with those obtained by direct injection of control samples (in 90/10 methanol/water, v/v) corresponding to the final concentrations of the extracted samples, assuming

100% recovery. Accuracy was determined by quantitating drug-free blood spiked with FK506 at 10 μg/L and 25 μg/L final concentrations ten times. Inter-assay precision was assessed by analysis of a 20 μg/L spiked drug-free blood pool, ten times over a twenty-day period. The inter-assay coefficient of variation (CV) was calculated (details and results are in chapter III).

### Extraction of FK506 metabolites from human blood specimens

#### Materials

Human FK506 trough blood specimen (blood collected right before patients taking the next dosage)- volunteer patients under FK506 therapy

Biohazard Hood- VWR Scientific, Canada.

FK506 internal standard 10 μg /ml stock- from previous preparation.

Diethylether- Sigma- Aldrich, Canada. HPLC grade

Acetic acid- Caledon Inc. Canada.

Bench top centrifuge- IEC Centra GP8, Fisher, Canada.

Shaker- Eberbach, Canada.

Vortex- Fisher, Canada.

Nitrogen tank- Praxair, Canada.

Methanol- Sigma - Aldrich, Canada. HPLC grade

Acetonitrile- Sigma- Aldrich, Canada. HPLC grade

### Method

A liquid-liquid solvent extraction technique was used for extracting FK506 and its metabolites from the blood matrix. In the biohazard hood, 1 mL of blood was spiked with 25 µL internal standard (1.5 mg/L) and 1 mL of 0.1 M acetic acid. The mixture was vortexed for 15 seconds and shaken in 10 mL of diethyl ether for 15 minutes. The ether layer was collected after centrifugation of 1800 ×g for 5 minutes and taken to dryness under a stream of nitrogen. The dried residues were reconstituted in 100 µL mobile phase (methanol/acetonitrile/water pH3, 27/30/43, v/v/v) for LC/MS analysis. The final volume of the sample was sufficient for two injections of LC/MS analysis.

## Extraction of FK506 metabolites from human urine specimens

### Materials

Human FK506 urine specimens- pooled urine from patients under FK506 therapy

Biohazard Hood- VWR Scientific, Canada.

FK506 internal standard- 10 µg /ml stock from previously described preparation.

Diethylether- Sigma- Aldrich, Canada. HPLC grade

Acetic acid- Caledon Inc. Canada.

Bench top centrifuge- IEC Centra GP8, Fisher, Canada.

Shaker- Eberbach, Canada.

Vortex- Fisher, Canada.

Nitrogen tank- Praxair, Canada.

Methanol- Sigma- Aldrich, Canada. HPLC grade

Acetonitrile- Sigma- Aldrich, Canada. HPLC grade

Sodium acetate- Sigma- Aldrich, Canada.

### Method

The extraction of FK506 and its metabolites from urine was very similar to the method used for blood samples. The volume of the urine needed for extraction was 25 mL so that the quantities of the metabolites would be large enough to be precisely detected by the electro-spray mass spectrometer. The urine was acidified with 5 mL of 0.1 M sodium acetate buffer, pH 4.95. The mixture was shaken with 60 mL of diethyl ether for 20 minutes. The ether layer was collected and taken to dryness under a stream of nitrogen. The dried residues were reconstituted in 150 μL of mobile phase for LC/MS analysis.

## *In vitro* generation of FK506 metabolites

### Materials

Rabbit livers- from healthy adult rabbits fed with 1 g/L phenobarbital for four days.

Phenobarbital- Powder, Sigma- Aldrich, Canada.

Grinder- Polytron, USA.

Potassium chloride- Sigma- Aldrich, Canada.

1 M sodium phosphate buffer (pH 7.4)- Sigma- Aldrich, Canada.

Magnesium chloride- anhydrous, Sigma- Aldrich, Canada.

Glucose-6-phosphate- Sigma- Aldrich, Canada.

NADP- Sigma- Aldrich, Canada.

1 N hydrogen chloride- Sigma- Aldrich, Canada.

$N_2$ tank- Praxair, Canada.

Ultracentrifuge- Beckman, USA

Bench-top centrifuge- IEC Centra GP8, Fisher, Canada.

Liquid chromatographic/Mass spectrometry (LC/MS) system- Series 1100, Hewlett Packard, Canada.

$C_{18}$ High performance liquid chromatography (HPLC) column- Nova-Pak, 3.9 ×150 mm, Waters, USA.

Diethylether- Sigma- Aldrich, Canada. HPLC grade

Acetic acid- Caledon Inc. Canada

Methanol- Sigma- Aldrich, Canada. HPLC grade

Acetonitrile- Sigma- Aldrich, Canada. HPLC grade

Balance- AG204, Mettler-Toledo Inc. Canada.

## Method

Phenobarbital (1g/L) was provided freely in the drinking water to male rabbits weighing around 1 Kg for four successive days to induce CYP 3A (Nelson et al., 1996; Parkinson, 1996). These rabbits were sacrified after 12-hour fasting. Their livers were removed, weighed and put into 0-4 °C 1.15% KCl immediately to maintain the enzyme activity. The blood clot and hemoglobulins were washed out with cold 1.15 % KCl. The livers were then cut into pieces and homogenized with 3 volumes of fresh cold 1.15% KCl with the grinder. The homogenate was centrifuged at 9000 ×g at 4 °C for 10 minutes. The supernatant was then ultra-centrifuged at 105000 ×g at 4 °C for 60 minutes. After ultra-centrifugation, the pellet (microsomes) was washed with cold 1.15% KCl solution and covered with 1 mL 0.1 M phosphate buffer (pH 7.4) for storage at – 70 °C. When studying FK506 metabolism in the liver microsomal system, the frozen rabbit liver microsomes were thawed on ice, centrifuged, weighed, and homogenized to make 600 mg/mL microsomes in 0.1 M phosphate buffer (PB, pH 7.4). 490 μL of 0.1 M sodium phosphate buffer (pH 7.4), 100 μL 50 mM $MgCl_2$ and 0.5 mM EDTA in PB, 100 μL 50 mM glucose-6-phosphate (G-6-P) in PB, and 100 μL 50 mM NADP in PB were mixed well and incubated at 37 °C for 5 minutes. 100 μL 600 mg/mL rabbit liver microsomes in 1.15% KCl were added to the reaction and incubated at 37 °C for 5 minutes again. Then 100 μL 10 unit/mL G-6-P dehydrogenase in PB, and 10 μL 10 mM FK506 in methanol or 10 μL methanol (control reaction) were added into reaction at 37 °C for various incubation times. The reactions were stopped by the addition of 40 μL 1 N HCl and vortexed well. That solution was then extracted. The procedure to extract the metabolites from the rabbit liver microsomal system was very similar to the one used for human blood samples, using diethylether as extracting solvent. The extract was put through the LC/MS system for the purposes of identification of FK506 metabolites, and a comparison of the profile of metabolites was made with the ones in human specimens. The identical metabolites were chosen and large quantities of these metabolites were purified from a rabbit liver microsomal generation system by collecting each metabolite from a HPLC system for further investigation. Various HPLC gradient systems were used in the process of purification of FK506 metabolites (done by colleagues in the lab). The purity of each metabolite was ensured by flow-injecting the final collection of the eluted peak into mass spectrometer at a flow rate of 50 μL/ min. By scanning all the mass range, only the spectrum of the metabolite should appear in the mass spectrograph.

### Investigation of possible phase II FK506 metabolites from human urine specimens

#### Materials

Drug- free human urine- volunteer from our laboratory

FK506 pooled urine- volunteers from the patients under FK506 therapy

Glucuronidase/sulfatase- Sigma- Aldrich, Canada.

Morphine and morphine-6-glucuronide mixture- In house preparation by spiking standards into urine. Standards were obtained from clinical toxicology laboratory at University of Alberta Hospital.

Water bath shaker- VWR Scientific, Canada.

$C_{18}$ sep-pak columns, VWR Scientific, Canada.

Glass tubes- Disposable 16 × 125 mm, VWR Scientific, Canada.

Liquid chromatographic/Mass spectrometry (LC/MS) system- Series 1100, Hewlett Packard, Canada.

$C_{18}$ High performance liquid chromatography (HPLC) column- Nova-Pak, 3.9 ×150 mm, Waters, USA.

Diethylether- Sigma - Aldrich, Canada. HPLC grade

Acetic acid- Caledon Inc. Canada

Methanol- Sigma- Aldrich, Canada. HPLC grade

Acetonitrile- Sigma- Aldrich, Canada. HPLC grade

pH meter- Accumet Basic, Fisher Scientific, Canada.

## Method

In order to investigate the possibility of formation of FK506 phase II metabolites (mainly glucuronide and sulfate-conjugated FK506) in urine, four groups of urine samples were used. Three of them were from patients' pooled urine. Of these three groups, one was for the negative control-1, the other was for positive control, and another was for the test. The fourth group was from normal drug-free urine and used as negative control-2. Each group was tested three times. A mixture containing 10 ng of morphine and 5 ng of morphine-6-glucuronide was spiked into the urine of the positive and negative control-1 groups. The purpose of spiking the morphine and morphine glucuronide into the control is to see if the enzyme activity of β-glucuronidase/sulfatase is efficient. The negative control-2 was used to see if the identified metabolites only showed up in FK506 patients' urine. 25 mL of urine from each group was adjusted to pH 5 by using 0.1 M sodium acetate buffer (pH 4.83). 20 μL of β-glucuronidase/sulfatase stock that made up 1000 or 5000 units of β-glucuronidase/sulfatase in the reaction was added to the positive control, the negative control-2, and the test groups. The purpose of adding various concentrations of β-glucuronidase/sulfatase was to ensure that the enzyme activity was high enough to initiate the reaction. 20 μL of water was added to the negative control-1. All the reaction mixtures were shaken in a water bath of 37 °C for 12 hours. Diethylether was added to the test and negative control-2 at the end of reaction for a liquid-liquid extraction. The positive control and negative control-1 (containing morphine/morphine glucuronide mixture) were extracted by a solid phase method ($C_{18}$ sep-pak cartridges) to extract out the morphine mixture. The cartridges were first pre-wetted with methanol (1mL), followed by water (1 mL) under 10-psi vacuum. The urine samples were then applied to the cartridges and washed with 4 mL of water. Morphine and morphine glucuronide were then eluted with 1 mL of methanol (Pawula, 1993). The LC/MS method used for detecting the morphine mixture was different from the one for FK506 metabolites. Briefly, the effluents from the sep-pak cartridge were put through a 150 mm C-18 HPLC column. The mobile phase contained 5% acetonitrile and 95% 50 mM sodium ammonium salt, pH 3.0. The flow rate was 0.6 mL/min from 0 min to 4 min, then gradually increased to 1.1 mL/min at 7 min. Morphine and morphine glucuronide were detected by mass spectrometry after the HPLC separation. The mass spectrum of morphine sodium-addup ion is 286 and of morphine glucuronide is 462 (in-house method, validated by another colleague).

## Results

Figure II- 1 shows the electrospray- mass spectrum of purified FK506. The mass of the FK506 sodium add-up ion is 826. The full range mass spectrum scan of the stock contained only the spectrum of FK506. This purified FK506 was prepared from commercially available FK506 capsules. The extraction method has been described in a previous section.

Figure II-2 A shows the LC/MS profile of acetyl-FK506 and FK506. The mass of the sodium add-up acetyl-FK506 ion is 868. Three peaks were identified as acetyl-FK506. The first and the third peak of acetyl-FK506 also contained FK506 tautomers. The second peak of acetyl-FK506 was chosen and purified as the internal standard for the purpose of the extraction of FK506 and its metabolites. This peak was collected and pooled into a glass flask. It was taken to dryness under a stream of nitrogen and dissolved in methanol to make a 10 μg /ml stock.

Figure II-2 B represents the chemical structure of acetyl-FK506.

Figure II-3 shows the electrospray-mass spectrum of purified acetyl-FK506. The mass of acetyl-FK506 is 868. The full range mass spectrum scan of the stock contained only the spectrum of acetyl- FK506. The acetylation procedure and purification of the product have been described in the previous section.

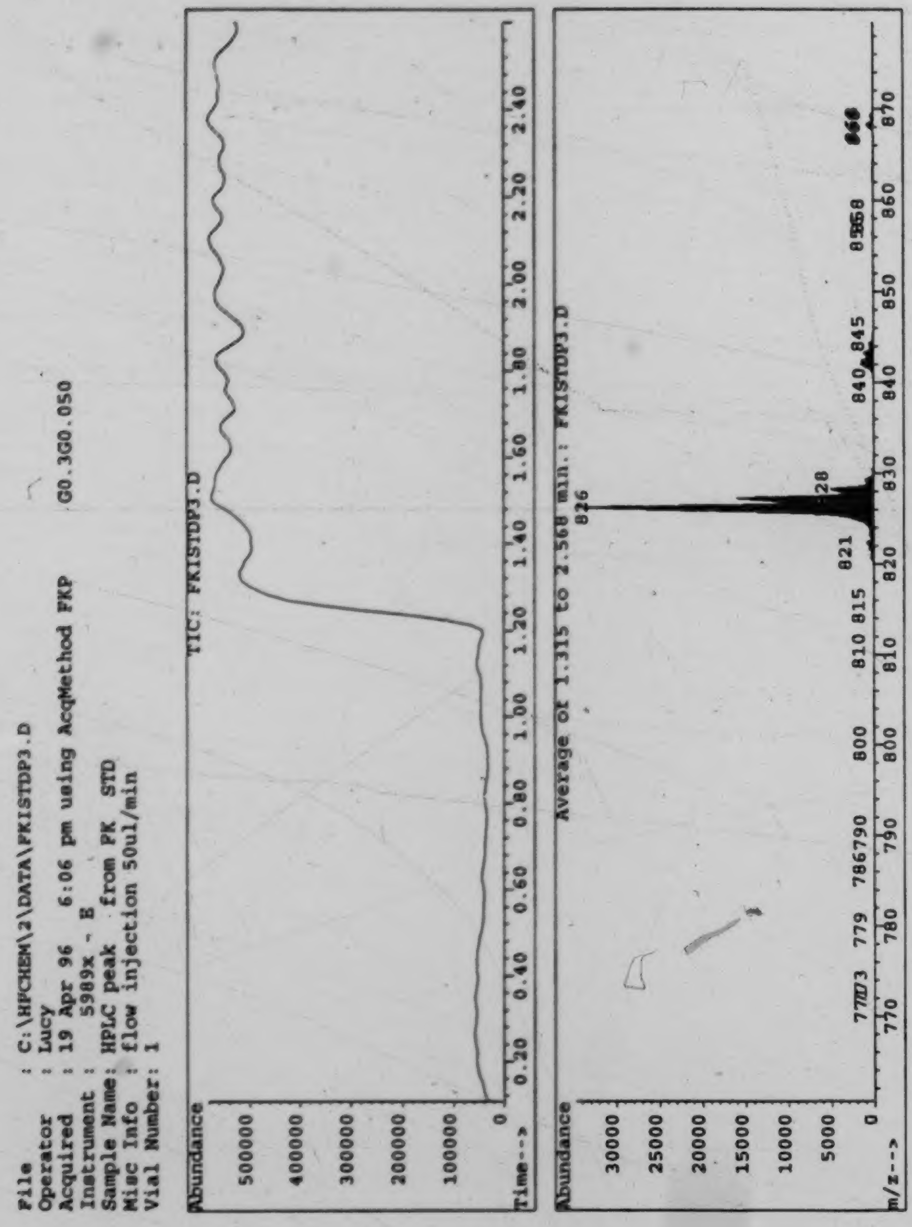
Figure II-4 compares the patterns of FK506 major metabolites between patients' blood, urine, the microbial metabolism system, and the rabbit liver microsomal system. FK506 metabolites were extracted from FK506-treated patients' pooled blood, pooled urine, rabbit liver microsomes, or the microbial (fungus) culture system. The establishment of a microbial system was done by other scientists in our laboratory. Actinoplanacete sp. ATCC no. 53771 was used in the fungus fermentation reaction. The incubation procedure was the same as Chen's method (Chen et al., 1991). The extraction procedures were identical for the samples from four different sources. The samples were run through the LC/MS system under identical conditions immediately after the extraction. There were eleven FK506 metabolites identified by LC/MS from different sources. The major metabolites in patients' blood and urine are 13-demethyl FK506 followed by 15-demethyl FK506. Although there is considerable 31-demethyl FK506 produced by the rabbit liver microsomal system and the fungus culture system, there was no evidence that it is in patients' blood and urine. The patterns of FK506 metabolites produced from these biological sources are summarized in table II-1. Generally speaking, the rabbit liver microsomal producing system and the fungus culture system produced more identifiable FK506 metabolites than the FK506 treated-patients' blood and urine. The rabbit liver microsomal system is also much cleaner than blood. Therefore, the rabbit liver microsomal system is a good source of large quantities of FK506 metabolites for further purification and investigation of the metabolites.

Figure II- 5 shows the electrospray-mass spectrum of purified 13-demethyl FK506, 15-demethyl FK506, and 31-demethyl FK506. All of them have masses of 812, which represent a sodium add-up demethylated FK506 ion.

Figure II- 6 shows the electrospray-mass spectrum of purified 13,31-didemethyl FK506 and 15,31-didemethyl FK506. Both of them have masses of 798.

Figure II- 7 shows the LC/MS profile of a morphine/morphine glucuronide mixture in urine before and after the enzymatic reaction with β glucuronidase/ sulfatase. The peak area of morphine glucuronide decreased and the peak area of morphine increased after enzymatic reaction.

Table II-2 shows the results of the investigation of possible phase II FK506 metabolites in urine. Two negative controls [patients' pooled urine with no β glucuronidase/sulfatase (I) and normal drug-free urine (II)], one positive control (patients' pooled urine spiked with morphine/morphine glucuronide) and two test groups (patients' pooled urine with 1000 or 5000 units of β glucuronidase/sulfatase) underwent incubation (pH 5, 30 °C, 12 hours). The final samples were extracted by different methods, depending on the targets that need to be extracted (FK506 metabolites or Morphine mixture). All the extracts were run by LC/MS under identical conditions. The concentrations of various metabolites were calculated to see if there was any difference between the mixture with the enzymes present and those with the enzymes absent. The results were compared by a t-test. There was no significant difference between negative control II and the test groups (p values > 0.05), indicating that there is no evidence that the phase II FK506 metabolites exist.



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Instrument  :    5989x - B
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Vial Number: 1
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Figure II- 1: The electrospray mass spectrum of purified FK506.

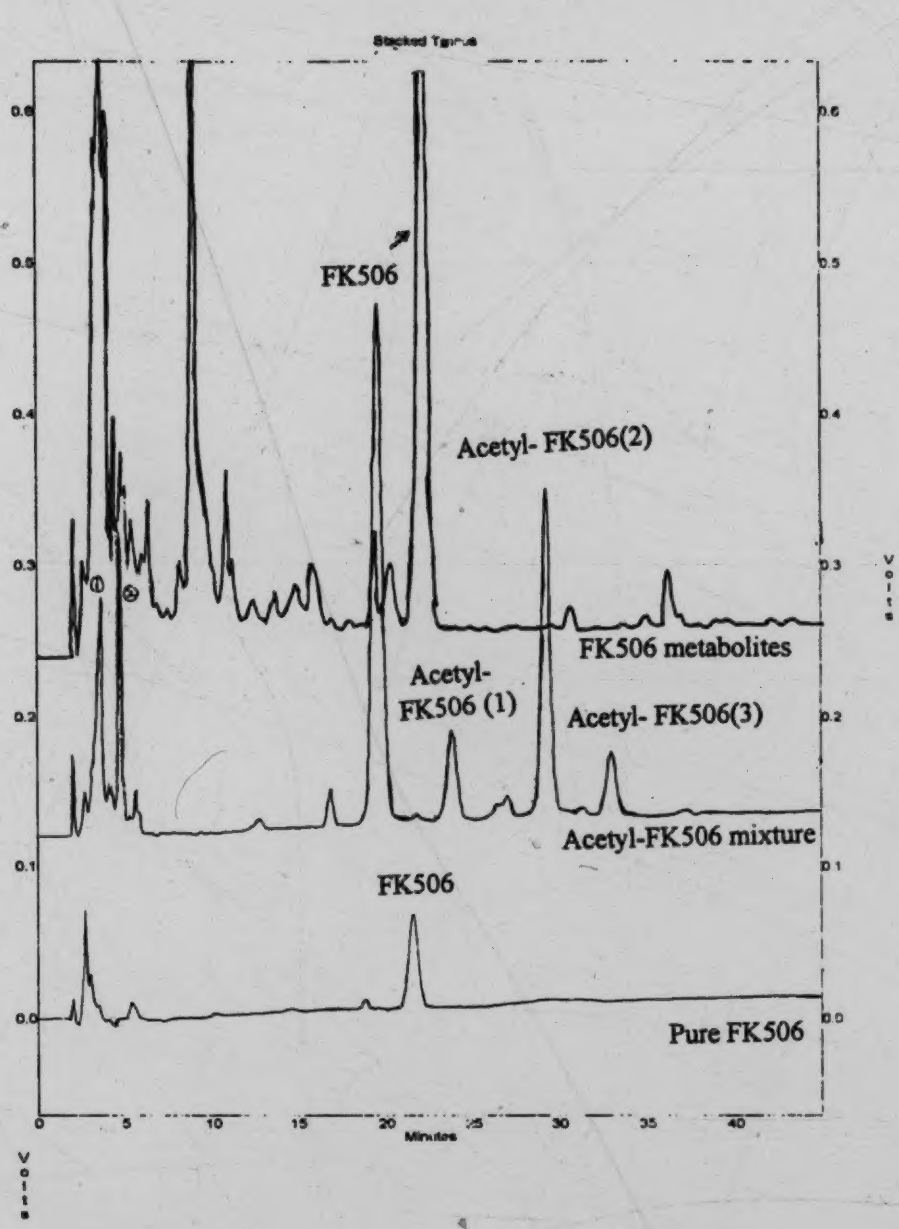
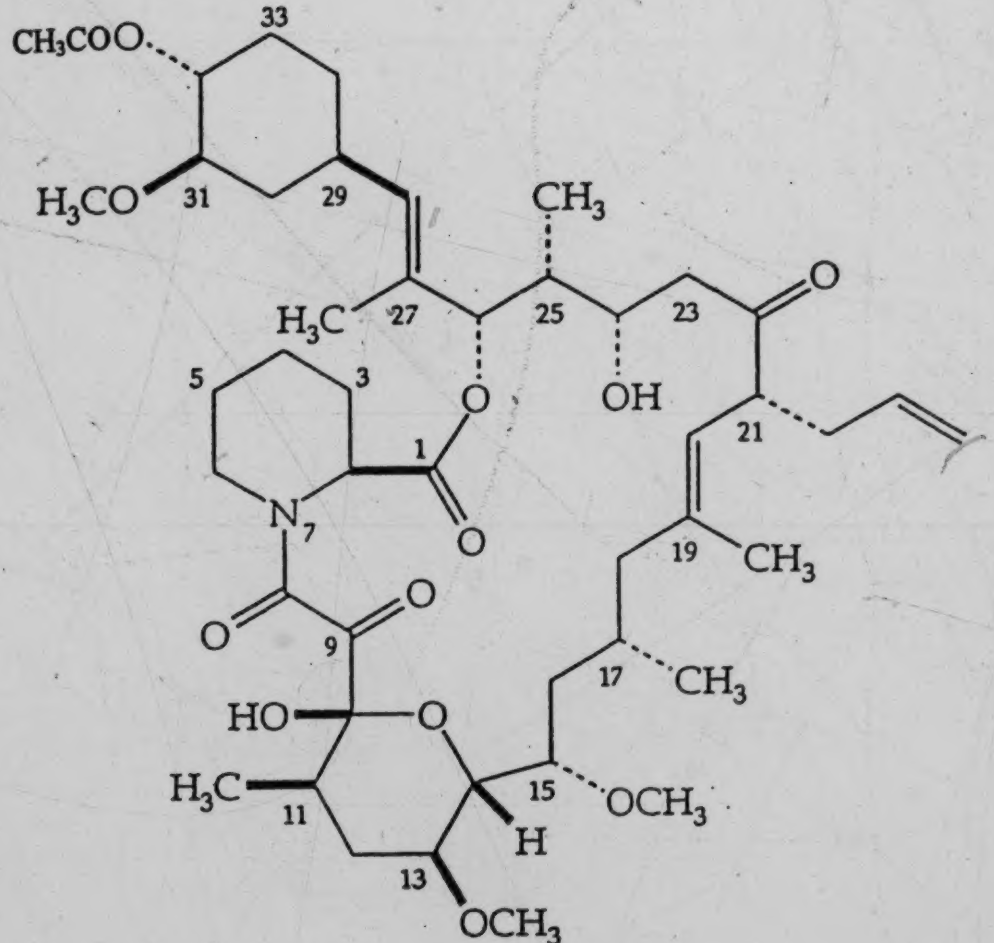


Figure II-2 A: LC/MS profile of acetyl-FK506 and FK506.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H3CO
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29
CH3
H3C
27
25
23
O
5
3
O
OH
1
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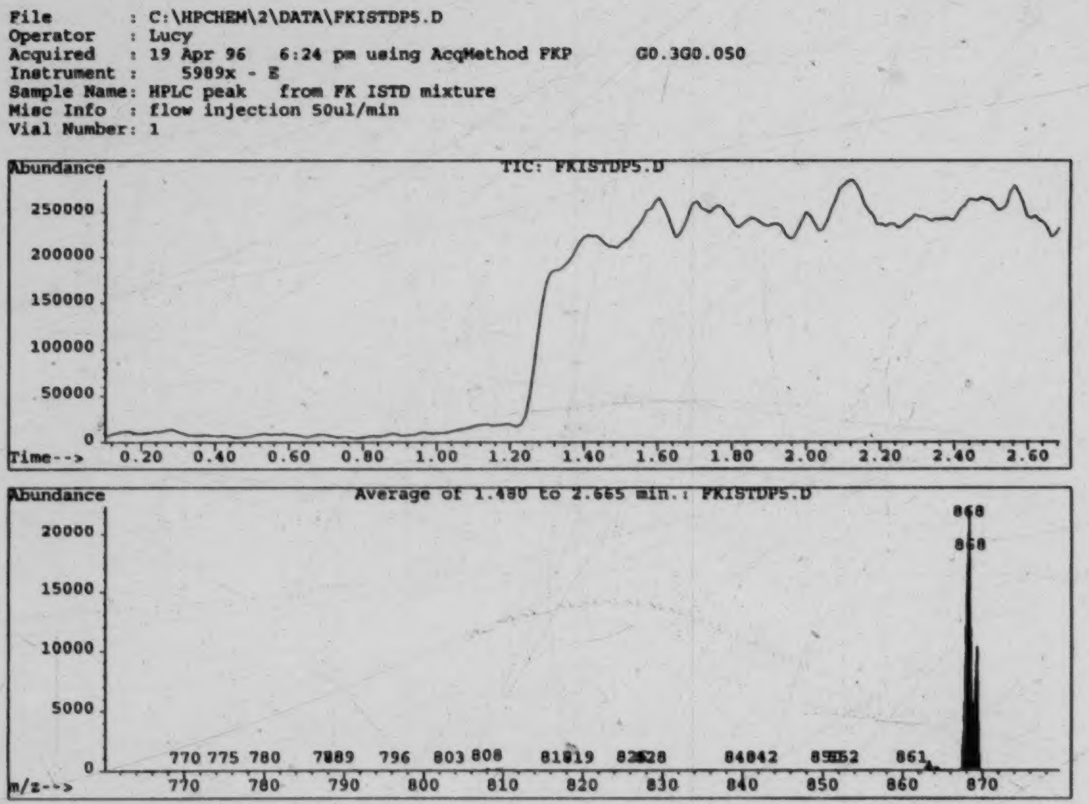
O
19
CH3
O
O
9
17
CH3
HO
O
H3C
11
15
OCH3
H
13
OCH3

Figure II-2 B: The chemical structure of acetyl-FK506.



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Operator    : Lucy
Acquired    : 19 Apr 96   6:24 pm using AcqMethod FKP      G0.3G0.050
Instrument  :    5989x - E
Sample Name: HPLC peak   from FK ISTD mixture
Misc Info   : flow injection 50ul/min
Vial Number: 1

Figure II- 3: The electrospray mass spectrum of purified acetyl- FK506.

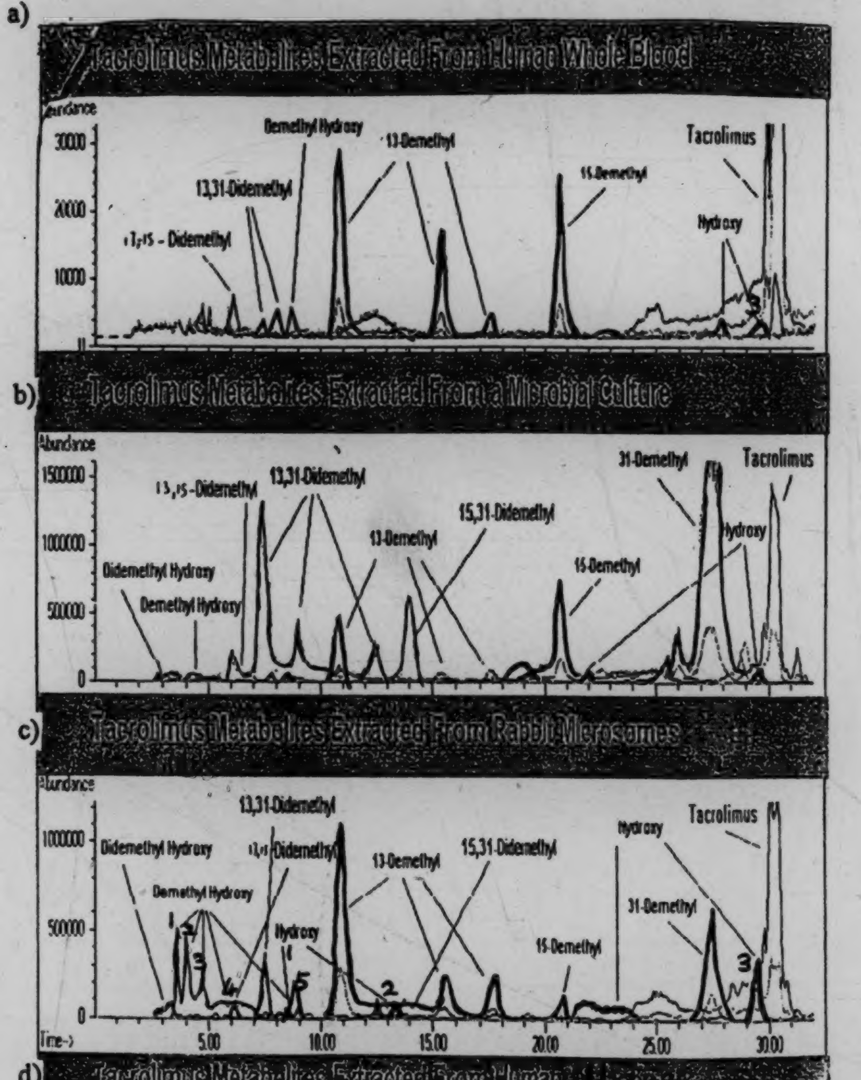
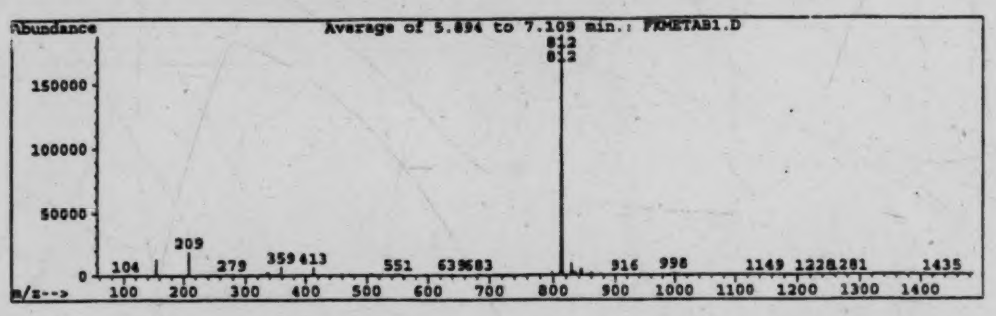
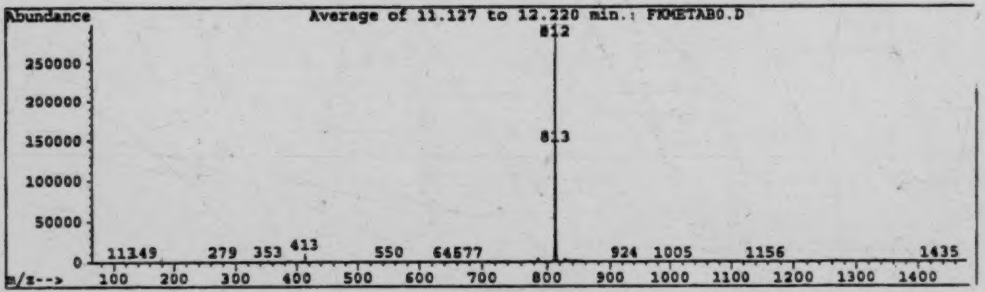


Figure II-4: LC/MS profiles of FK506 and its metabolites extracted from a) human whole blood, b) microbial culture, c) rabbit liver microsomal system, and d) human urine.

ESI-MS Spectrum (100-1500 amu) of 13-demethyl Tacrolimus



ESI-MS Spectrum (100-1500 amu) of 15-demethyl Tacrolimus



ESI-MS Spectrum (100-1500 amu) of 31-demethyl Tacrolimus

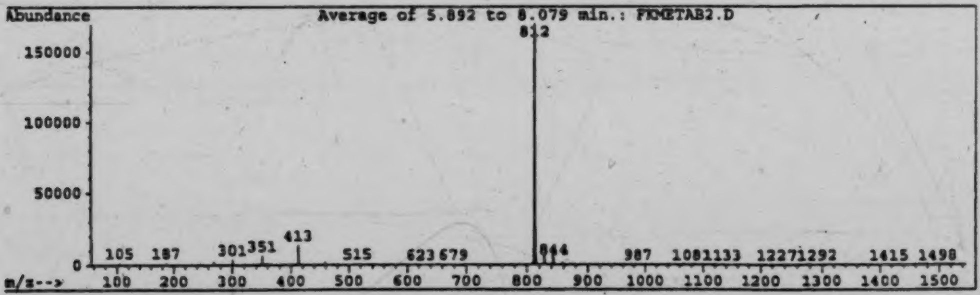
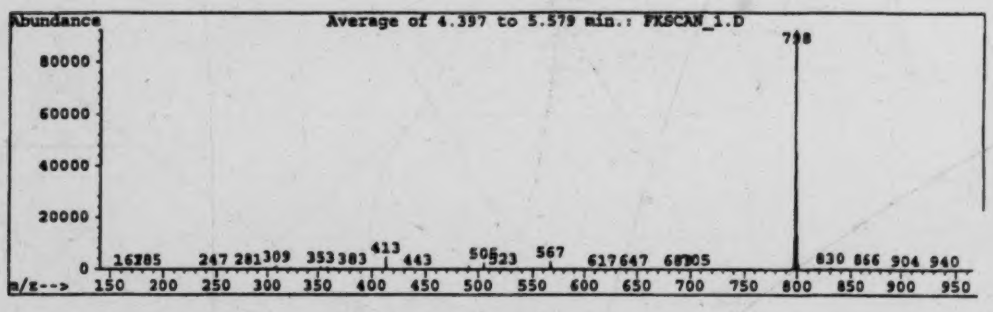


Figure II- 5: The electrospray mass spectrum of purified 13- demethyl FK506, 15- demethyl FK506, and 31- demethyl FK506.

Mass Spectrum (100-1500 amu) of 13, 31-Didemethyl Tacrolimus



Mass Spectrum (100-1500 amu) of 15, 31-Didemethyl Tacrolimus

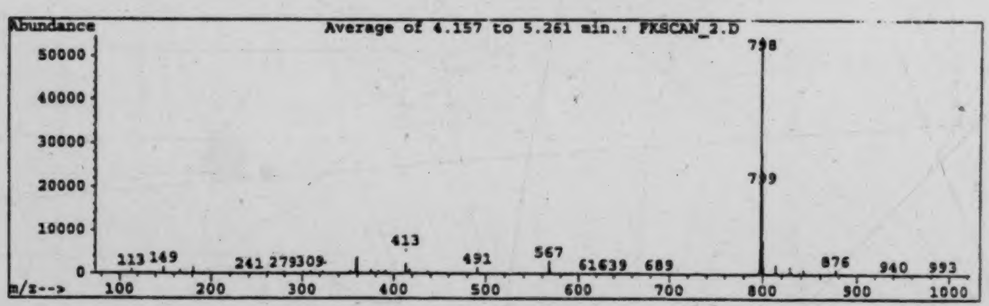
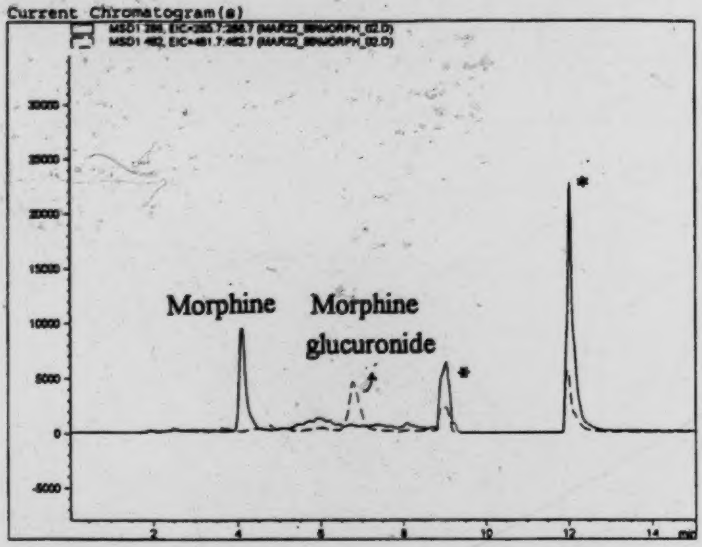


Figure II- 6: The electrospray mass spectrum of purified 13,31-didemethyl FK506 and 15,31-didemethyl FK506

**Before**



**After**

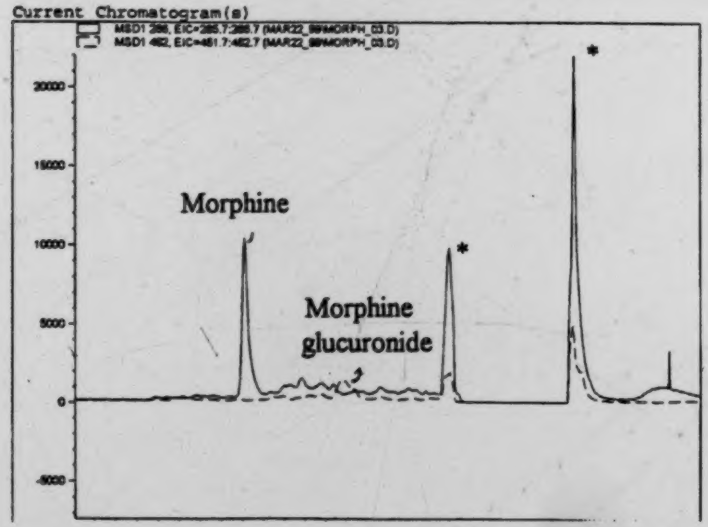


Figure II-7: LC/MS profile of morphine/morphine glucuronide mixture in urine before (top) and after (bottom) the enzymatic reaction with β glucuronidase/sulfatase. * = unidentified peak.

| FK506 metabolites | Human blood | Microbial culture | Rabbit liver microsome | Human urine |
|---|---|---|---|---|
| FK506 | + | + | + | + |
| 13-demethyl | + | + | + | + |
| 15-demethyl | + | + | + | + |
| 31-demethyl | - | + | + | - |
| 13,15-didemethyl | + | + | + | - |
| 13,31-didemethyl | + | + | + | + |
| 15,31-didemethyl | - | + | + | - |
| Demethyl-hydroxyl (1) | - | - | + | - |
| Demethyl-hydroxyl (2) | - | - | + | - |
| Demethyl-hydroxyl (3) | - | + | + | - |
| Demethyl-hydroxyl (4) | + | - | + | - |
| Demethyl-hydroxyl (5) | - | - | + | - |
| Didemethyl-hydroxyl | - | + | + | - |
| Dihydroxyl | - | - | - | + |
| Hydroxyl (1) | - | - | + | - |
| Hydroxyl (2) | + | + | + | + |
| Hydroxyl (3) | + | + | + | - |

Table II-1: Summary of FK506 metabolite patterns from various biological sources.

"+" Represents levels > 0.1 µg/L whereas "-" represents levels < 0.1 µg/L

| p | 13 demethyl- FK506 | 15 demethyl-FK506 | Hydroxy FK506 |
|---|---|---|---|
| Negative I vs. test I | 0.143 | 0.259 | 0.927 |
| Negative I vs. test II | 0.588 | 0.061 | 0.436 |
| Test I vs. test II | 0.399 | 0.18 | 0.493 |

Table II-2: t- test results from the investigation of possible phase II FK506 metabolites in urine. The results were described in page 55.

# Chapter III

## Comparison of methods used to monitor trough FK506 blood concentrations

### Objective

The purpose of this study is to investigate the reliabilities of the current analytical methods (immunoassays) used to monitor trough blood FK506 concentrations. It was assumed that the cross-reactivity of FK506 metabolites with the anti-FK506 antibody in the immunoassay would influence the results of FK506 concentrations measured by the immunoassays. The experiment focused on comparing the routine immunoassays i.e. the enzyme linked immunosorbent assay (ELISA) and the micro-particle enzyme immunoassay (MEIA) with an analytical reference assay utilizing liquid chromatography/mass spectrometry (LC/MS). Trough blood samples from FK506 treated patients were chosen randomly from the transplant patients under FK506 therapy at the University of Alberta Hospital.

## Materials and Methods

### FK506 enzyme linked immunosorbent assay (ELISA)

### Materials

Two sets of FK506-treated patients' trough blood samples (73 and 48) in three groups (from patients undergoing liver, kidney, or heart-lung transplantation)

Pro-Trac II FK506 Enzyme-Linked-Immunosorbent-assay (ELISA) kits- INCSTAR Co. USA

12 × 75 mm conical bottom tubes- VWR Scientific, Canada.

Double deionized water

Blood rotator- Fisher, Canada.

Tube shaker- Fisher, Canada.

Water bath- VWR Scientific, Canada.

Plate shaker- VWR Scientific, Canada.

Timer- Fisher, Canada.

Microplate spectrometer- VWR Scientific, Canada.

Ice bucket- VWR Scientific, Canada.

Ice machine- VWR Scientific, Canada.

Bench top centrifuge- IEC Centra GP8, Fisher, Canada.

Micro pipette (1-10 μL, 10-50 μL, 100-1000 μL)- Nichipet, Canada.

Micro pipette tips- VWR Scientific, Canada.

## Methods

The FK506 Pro-Trac II ELISA developed by INCSTAR Co. is a sensitive competitive immunoassay utilizing a monoclonal antibody against FK506 to measure FK506 concentrations in biological fluids. This assay is run in a micro-titer plate pre-coated with goat anti-mouse IgG. 50 μL of each standard (including non-specific binding, NSB), control (3 and 15 μg/L), or samples (blood) were pipetted into the appropriately labeled tube. Special formulated digestion reagent (300 μL) was then added each tube. All tubes were covered and vortexed for 15- 30 seconds. After incubation at room temperature for 15 ± 2 minutes, the tubes were transferred into a 75 ± 1 °C water bath for 15 ± 2 minutes. The tubes were then removed from the water, vortexed, and centrifuged for 10 minutes at 1800 × g at room temperature. Supernatant (100 μL) from each tube was pipetted into the wells of the micro-titer plate in duplicate. Anti-FK506 monoclonal antibody (50 μL) was pipetted into all the wells except the NSB ones. Conjugate diluent (50 μL) was pipetted into the NSB wells. The plate was covered with parafilm and a plate cover and fastened to a plate shaker which was shaken for 30 ± 2 minutes at 700 ± 50 rpm and room temperature. After 30 minutes of shaking, diluted conjugate concentrate (50 μL) was added into each well. The plate was covered again and shaken for another 60 ± 5 minutes under the same conditions. At the end of shaking, the wells were washed three times with dilute washing solution. The plate was inverted and pounded against absorbent paper to get rid of residual washing solution. Chromogen (200 μL)was pipetted into each well within 5 minutes after washing the plate. The plate was covered again and shaken for 15 ± 1 minutes at 700 ± 50 rpm and room temperature. Stop solution (100 μL) was vigorously pipetted into each well at the end of shaking. The color of the solution in the wells should change from blue to yellow. The plate was read on a micro-titer plate reader at 450/650 nm dual wavelengths within 5 minutes after adding the stopping solution. The "4PL curve fitting" program from the software of the plate reader was used to plot absorbency vs. log concentration of standards to interpolate the concentrations in the controls and samples. The results (in μg/L) of all blood samples were recorded for later comparison.

### FK506 IMx micro-particle enzyme immunoassay (MEIA)

#### Materials

FK506 IMx Micro-particle-Enzymatic-Immunoassay (MEIA) kits- Abbott Laboratories, Abbott park, IL, USA

48 FK506 treated patients' trough blood samples- patients from University of Alberta Hospital.

Blood rotator- Fisher, Canada.

Glass precision pipette (100 μL, 200 μL)- Fisher, Canada.

Bench top centrifuge- ICE Centra GP8, Fisher, Canada.

Vortex- Fisher, Canada.

IMx automatic analyzer– Abbott Laboratories, Abbott park, IL, USA

Deionized water

### Method

The FK506 MEIA developed by Abbott Laboratories, USA, is another immunoassay used to monitor FK506 concentrations clinically. It utilizes the same anti-FK506 antibody used in ELISA and runs in an automated IMx instrument from Abbott. Prior to the initiation of the automatic IMx sequence, a manual pretreatment step was performed for whole blood samples. Briefly, 100 µL of each sample, calibrator, or control were pipetted into a ×- systems centrifuge tube. Then, 200 µL of IMx FK506 whole blood precipitation reagent was pipetted into each centrifuge tube. The tubes were capped immediately and vigorously vortexed for 10-15 seconds. These tubes were then loaded into a ×-systems centrifuge rotor and centrifuged at 1800 ×g for 2 minutes. 150 µL of each supernatant was withdrawn immediately after the centrifugation into the sample well of an IMx reaction cell. All the cells were then put into the IMx machine for the automatically analytic procedure. The sequence is as follows: The probe/electrode assembly delivers the sample and anti-FK506-coated micro-particles to the incubation well of the reaction cell. FK506 binds to anti-FK506-coated micro-particles, forming an "antibody-antigen" complex. An aliquot of the reaction mixture containing these complexed micro-particles is transferred to the glass fiber matrix to which the micro-particles can bind irreversibly. The FK506/ alkaline phosphatase conjugate is dispensed onto the matrix and binds to the available sites on the anti-FK506-coated micro-particles. The matrix is washed to remove unbound materials. The substrate, 4-methylumbelliferyl phosphatate, is added to the matrix and the fluorescent product is measured by the MEIA optical assembly. The blood samples that were measured by FK506 ELISA were also measured by MEIA. The results (in μg/L) were recorded for further analysis.

LC/MS assay

Materials

FK506 standards- in-house prepared

Acetyl-FK506 (in-house internal standard)

48 FK506 treated patients' trough blood samples

Liquid chromatography/Mass spectrometry (LC/MS) system- Series 1100, Hewlett Packard, Canada.

$C_{18}$ High performance liquid chromatography (HPLC) column- Nova-Pak, 3.9 ×150 mm, Waters, USA

Diethylether- Sigma- Aldrich, Canada. HPLC grade

Acetic acid- Caledon Inc. Canada.

Methanol- Sigma- Aldrich, Canada. HPLC grade

Acetonitrile- Sigma- Aldrich, Canada. HPLC grade

## Method

The procedure for preparation of FK506 standards was as follows: 5, 10, 15, 20 or 30 μL of FK506 stock 10 μg/mL in methanol were spiked into empty tubes. The tubes were brought to dryness under a stream of nitrogen. 10 mL of drug-free blood was then spiked into each tube to make 5, 10, 15, 20, or 25 μg/L standards respectively. An LC/MS system was developed as a reference method to monitor FK506. Two tandem $C_{18}$ HPLC columns heated at 50°C were used to optimize the HPLC separation of FK506 and its metabolites. FK506 standards or extracts from the biological sources were first injected into the HPLC system and separated by using a methanol (MeOH)/ acetonitrile (ACN)/acetic water (pH 3) gradient at a flow rate of 1.25 mL/min. The gradient profile was as follows: 0-15 minutes: 27% MeOH/30% ACN/43% pH 3 water → at 25 minutes: 40% MeOH/30% ACN/30% pH 3 water → at 30 minutes: 60% MeOH/30% ACN/10% pH 3 water → 35 minutes: 100% MeOH. The HPLC column effluent was then introduced into the mass spectrometer via an electrospray interface. The instrument was operated in selected ion monitoring mode. The mass of the ion to be monitored was chosen according to the mass of sodium add-up FK506. The concentration of FK506 was calculated according to a standard curve established by running standards together with the samples. Between-run precision and accuracy were determined from quality control (QC) samples spiked with three different concentrations of FK506. The concentrations of three quality controls used for the evaluation were 7.5 μg/L, 15 μg/L, and 25 μg/L respectively. A total of ten replicates of each QC concentration for FK506 were analyzed on five different dates. The concentrations of QCs were also determined by six different calibration curves from six different dates. Precision was expressed as the coefficient of variation (CV, %) while accuracy was measured as analytical recovery according to the following equation:

Analytical recovery = $[C_{Exp}/C_{Thr}] \times 100\%$

$C_{exp}$ = Mean concentration determined experimentally

$C_{Thr}$ = Theoretical concentration

### Comparison of immunoassays with LC/MS assay

Materials

Microcal Origin software- Microcal, Inc.

Computer- Pentium II 300 MHz, Gateway, Canada.

Method

A. Each of 73 FK506-treated patients' EDTA anti-coagulated blood samples (52 renal, 14 liver, 7 heart-lung transplantation) was separated into two tubes on the same day as withdrawn from the patients. Both groups of the tubes were frozen immediately until further analyzed by ELISA and LC/MS. The results of FK506 trough blood concentrations from ELISA and LC/MS were compared by linear regression using "Microcal Origin" software. The equation and "r" value for the regression were calculated to assess the relationship between the two assays.

B. 48 FK506 patients' trough blood samples were chosen randomly and analyzed by MEIA and ELISA. The results were compared under linear regression using "Microcal Origin" software. The equation and "r" value for the regression were calculated to assess the relationship between the two assays.

Note: Insufficient volumes of specimens prevented me to do the three assays in the same time.

## Results

The calibration curves of LC/MS for FK506 in human whole blood from 6 different dates are summarized in table III- 1. The C.V.s of FK506 were low with percentages ranging from 0.8 % to 7.8 %. The between-run precision and accuracy of the LC/MS assay are summarized in table III- 2. The C.V.s for FK506 were also low, with percentages ranging from 3.8% to 4.6%. The analytical recovery obtained for the 7.5 µg/L of FK506 (LQC), 15 µg/L of FK506 (MQC), and 25 µg/L of FK506 (HQC) were 92.4%, 95.2% and 92.8% respectively. The within-run precision and accuracy of the LC/MS assay are summarized in table III-3. Within-run precision and accuracy was determined similarly to between-run using a total of six replicates of the LQC, MQC, and HQC whose concentrations were determined from one calibration curve. The C.V.s ranged from 4.0 % to 5.9 %. The analytical recovery obtained for the QCs were 94.6%, 95.8%, and 97.7% respectively. The extraction efficiencies of

FK506 and acetyl- FK506 (ISTD) are summarized in table III-4. The extraction yields of FK506 in whole blood were 107.4% and 107.9% at 7.5 μg/L and 25 μg/L respectively. The extraction yield of acetyl- FK506 was 115%. Figure III-1 shows a typical FK506 calibration curve used in the LC/MS assay. The lowest quantitation limit of this LC/MS assay was 2 μg/L for 1 mL of blood sample.

Figure III-2 shows the results of 73 FK506-treated patients' trough blood samples (52 renal transplants, 14 liver transplants, and 7 heart-lung transplants) analyzed by both LC/MS and ELISA. The samples were chosen randomly. The purpose was to check if there was any bias between the two assays. After the comparison, the linear regression between two sets of results was determined by using Microcal origin. The equation for the regression is as follows: ELISA= 0.39+ 0.9997 × LC/MS. The r value for the regression was 0.957. The mean FK506 concentration measured by LC/MS was 10.75 ± 5.196 while the mean value measured by ELISA was 11.14 ± 5.427. ELISA has a positive mean bias of 0.39 μg/L compared with LC/MS (figure III-3).

Figure III-4 shows the results from 48 randomly chosen FK506-treated patients' trough blood samples analyzed by both MEIA and ELISA. The purpose was to check if there was any bias between the two assays. After the comparison, the linear regression between the two sets of results was determined by using Microcal origin. The equation for the regression is as follows: ELISA= -0.11 + 0.86 × MEIA. The r value for the regression is 0.94. The mean FK506 concentration measured by MEIA was 13.72 ± 4.28 while the mean value measured by ELISA was 11.67 ± 3.89. MEIA has a positive mean bias of 2.05 μg/L compared with ELISA (figure III-5).

| Standards | 5 ng/mL Tacrolimus | 10 ng/mL Tacrolimus | 20 ng/mL Tacrolimus | 30 ng/mL Tacrolimus | Correlation coefficient, r |
|---|---|---|---|---|---|
| Curve # 1 (Sept 28) | 5.21 | 9.73 | 20.02 | 30.04 | 0.9998 |
| Curve # 2 (Sept 30) | 4.56 | 11.06 | 18.97 | 30.40 | 0.997 |
| Curve # 3 (Oct 1) | 4.9 | 10.0 | 20.1 | 29.9 | 0.9999 |
| Curve # 4 (Oct 2) | 4.70 | 10.56 | 19.63 | 30.11 | 0.9992 |
| Curve # 5 (Oct 5) | 4.0 | 11.1 | 20.29 | 29.59 | 0.996 |
| Curve # 6 (Oct 6) | 4.7 | 10.5 | 19.81 | 30.01 | 0.9995 |
| | | | | | |
| Mean | 4.68 | 10.49 | 19.8 | 30.01 | |
| Standard Deviation | 0.367 | 0.50 | 0.427 | 0.24 | |
| Coefficient of Variation | 7.8 % | 4.8 % | 2.2 % | 0.8 % | |
| n | 6 | 6 | 6 | | |
| | | | | | |

Table III- 1: Calibration curve summary for FK506 in human whole blood.

|  | Low QC (7.5 ng/mL) | Med QC (15 ng/mL) | High QC (25 ng/mL) |
|---|---|---|---|
| Day 1 | 7.0 | 15.8 | 28.8 |
|  | 7.7 | 16.3 | 28.3 |
|  |  |  |  |
| Day 2 | 8.0 | 16.0 | 27.1 |
|  | 8.0 | 16.5 | 27.1 |
|  |  |  |  |
| Day 3 | 8.1 | 16.2 | 26.4 |
|  | 8.1 | 16.1 | 26.2 |
|  |  |  |  |
| Day 4 | 7.7 | 16.2 | 27.2 |
|  | 7.7 | 15.3 | 26.9 |
|  |  |  |  |
| Day 5 | 7.5 | 14.7 | 26.5 |
|  | 7.2 | 14.5 | 24.9 |
|  |  |  |  |
| Mean | 7.7 | 15.76 | 26.94 |
| Standard Deviation | 0.36 | 0.66 | 1.03 |
| Coefficient of Variation | 4.6 % | 4.1 % | 3.8 % |
| n | 10 | 10 | 10 |
|  |  |  |  |
| Accuracy (%) | 97.4 % | 95.2 % | 92.8 % |

**Table III- 2: Between-run precision and accuracy of LC/ MS assay.**

|  | Low QC (7.5 ng/mL) | Med QC (15 ng/mL) | High QC (25 ng/mL) |
|---|---|---|---|
|  | 7.8 | 15.8 | 23.0 |
|  | 8.0 | 16.5 | 26.8 |
|  | 8.7 | 14.6 | 25.0 |
|  | 7.7 | 16.2 | 25.1 |
|  | 8.1 | 15.5 | 22.6 |
|  | 7.2 | 15.3 | 24.1 |
|  |  |  |  |
| **Mean** | 7.93 | 15.65 | 24.43 |
| **Standard Deviation** | 0.446 | 0.62 | 1.41 |
| **Coefficient of Variation** | 5.9% | 4.0% | 5.8% |
| **n** | 6 | 6 | 6 |
|  |  |  |  |
| **Accuracy (%)** | 94.6 % | 95.8 % | 97.7 % |
|  |  |  |  |

Table III- 3: Within–run precision and accuracy of LC/ MS assay.

**Extraction Yields of Tacrolimus from Human Whole Blood**

| Peak Area | Low QC (7.5 ng/mL) Extracted | Low QC Unextracted | High QC (25 ng/mL) Extracted | High QC Unextracted |
|---|---|---|---|---|
| | 1978664 | 2020905 | 6762099 | 5858781 |
| | 2309522 | 1827807 | 6375858 | 5804761 |
| | 1837656 | 1896203 | 7016708 | 6815316 |
| | 2012621 | 1920172 | 7278230 | 6432156 |
| | 2250222 | 2005962 | 7015723 | 7295103 |
| | | | | |
| **Mean** | 2077737 | 1934210 | 6889724 | 6441223 |
| **Standard deviation** | 176607 | 71565 | 303147 | |
| **Coefficient of Variaition** | 8.5% | 3.7% | 4.4 % | 8.9% |
| **n** | 5 | 5 | 5 | 5 |
| | | | | |
| **Mean Recovery (%)** | 107.4% | | 107.9% | |

**Extraction Yields of Acetyl-Tacrolimus from Human Whole Blood**

| Peak Area | Internal Standard (Extracted) | Internal Standard (Unextracted) |
|---|---|---|
| | 11938556 | 9879140 |
| | 11860846 | 10126009 |
| | 10793719 | 10192021 |
| | 11057637 | 9498783 |
| | | |
| **Mean** | 11412689 | 9923988 |
| **Standard deviation** | 496630 | 313828 |
| **Coefficient of Variaition** | 4.4 % | 3.2 % |
| **n** | 4 | 4 |
| | | |
| **Mean Recovery (%)** | 115 % | |

Table III- 4: The extraction efficiencies of FK506 and acetyl–FK506 from human whole blood.

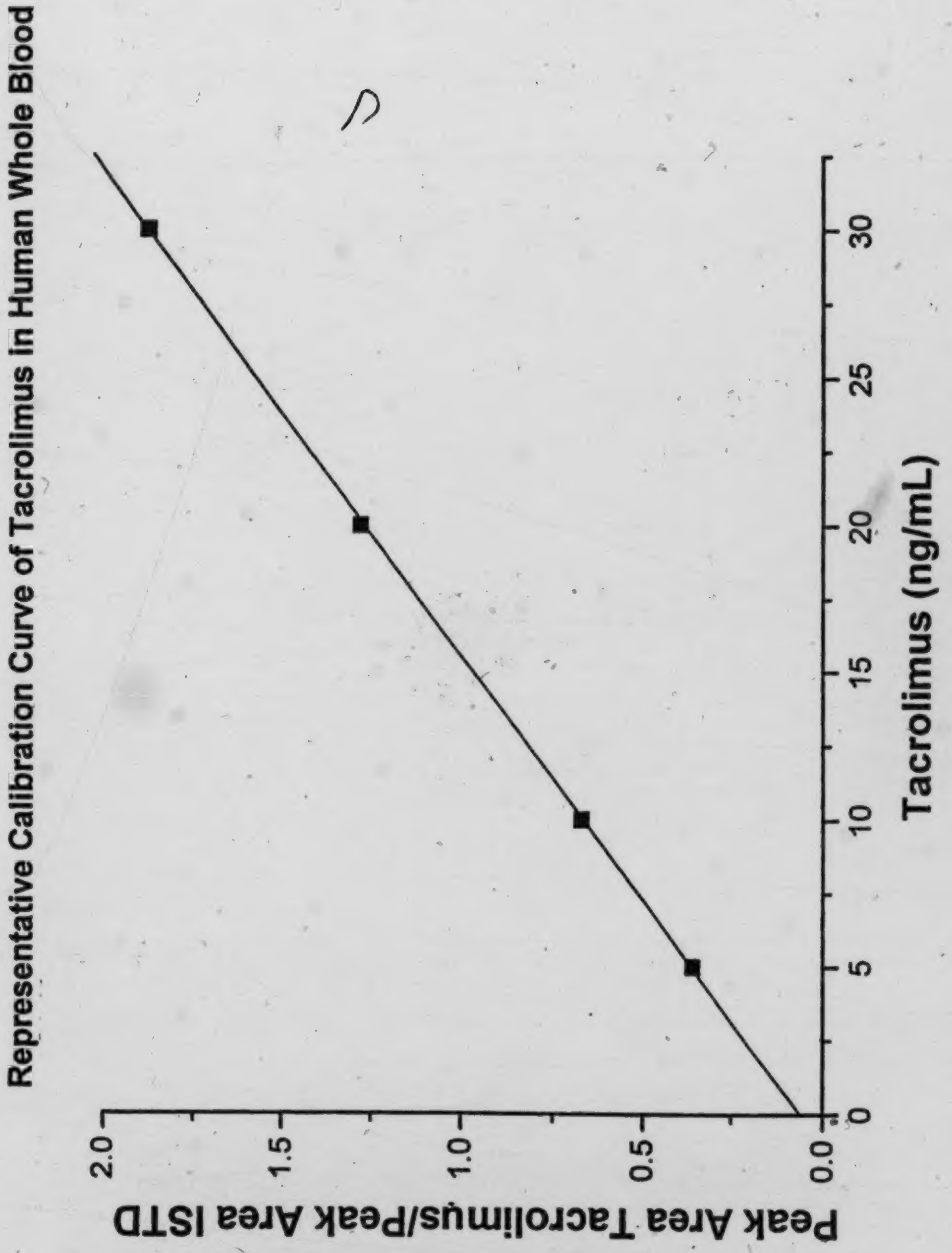


Figure III- 1: Representative calibration curve of FK506 in human whole blood measured by LC/MS assay.

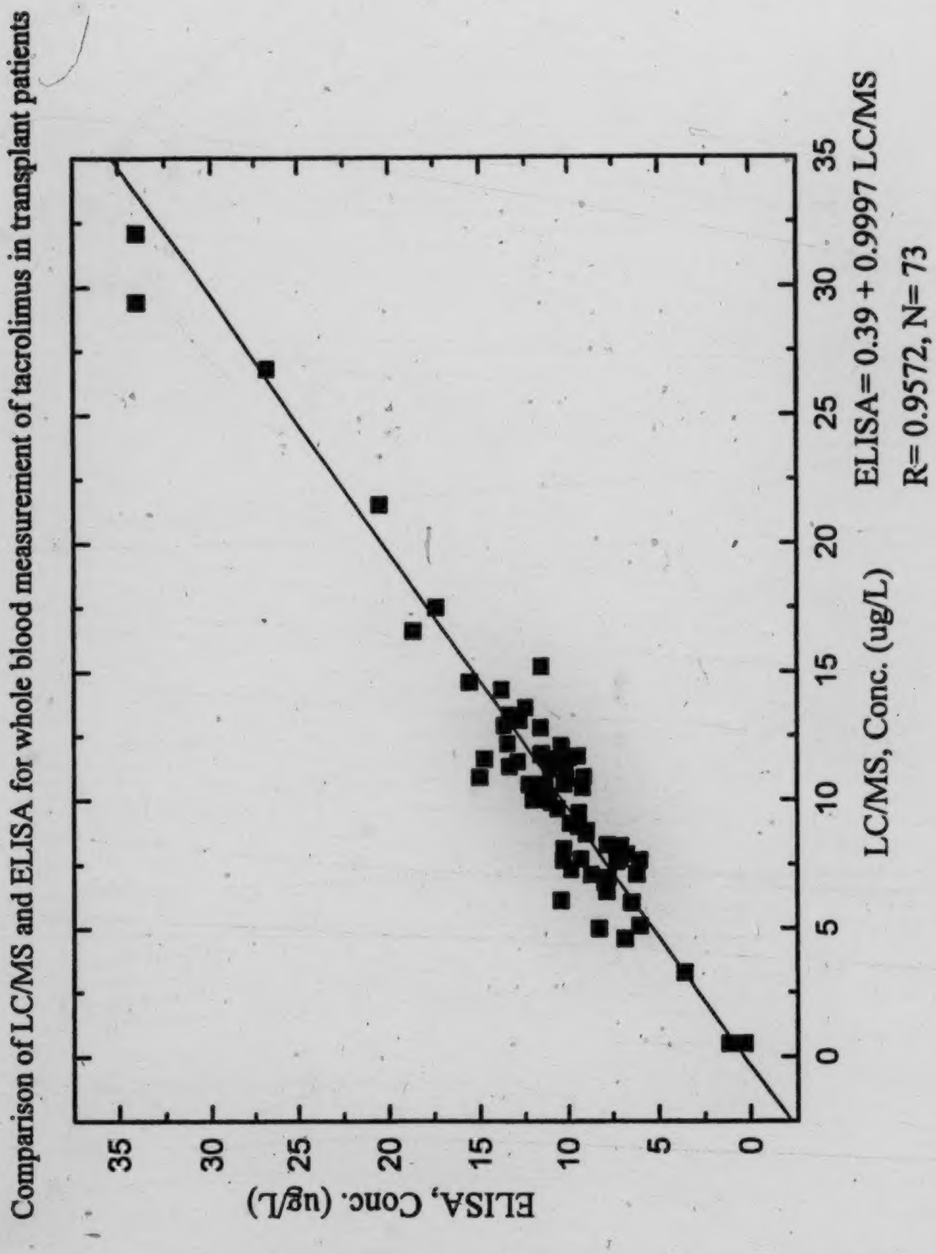


Figure III- 2: The linear regression for the results of FK506 trough blood concentration measured between LC/MS and ELISA

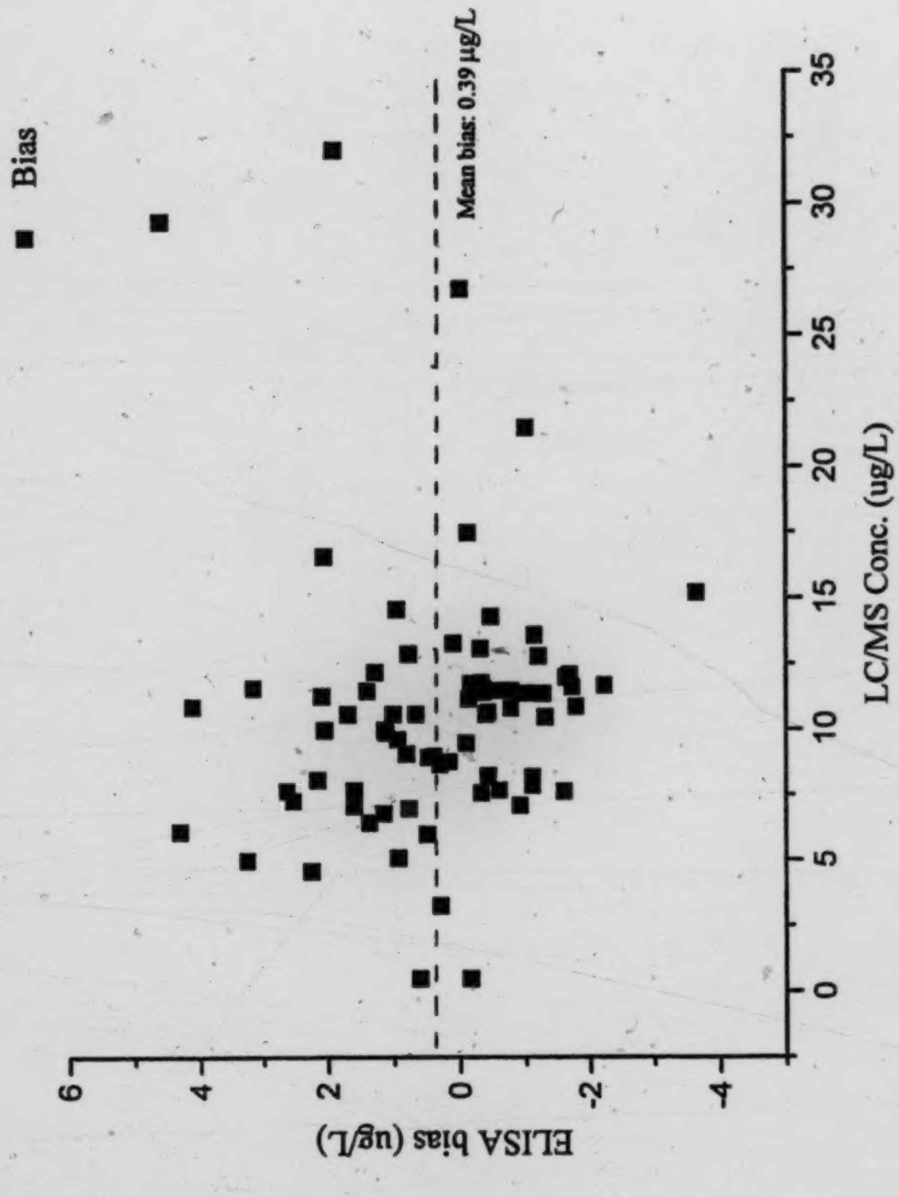


Figure III- 3: Bias plot between ELISA and LC/MS.

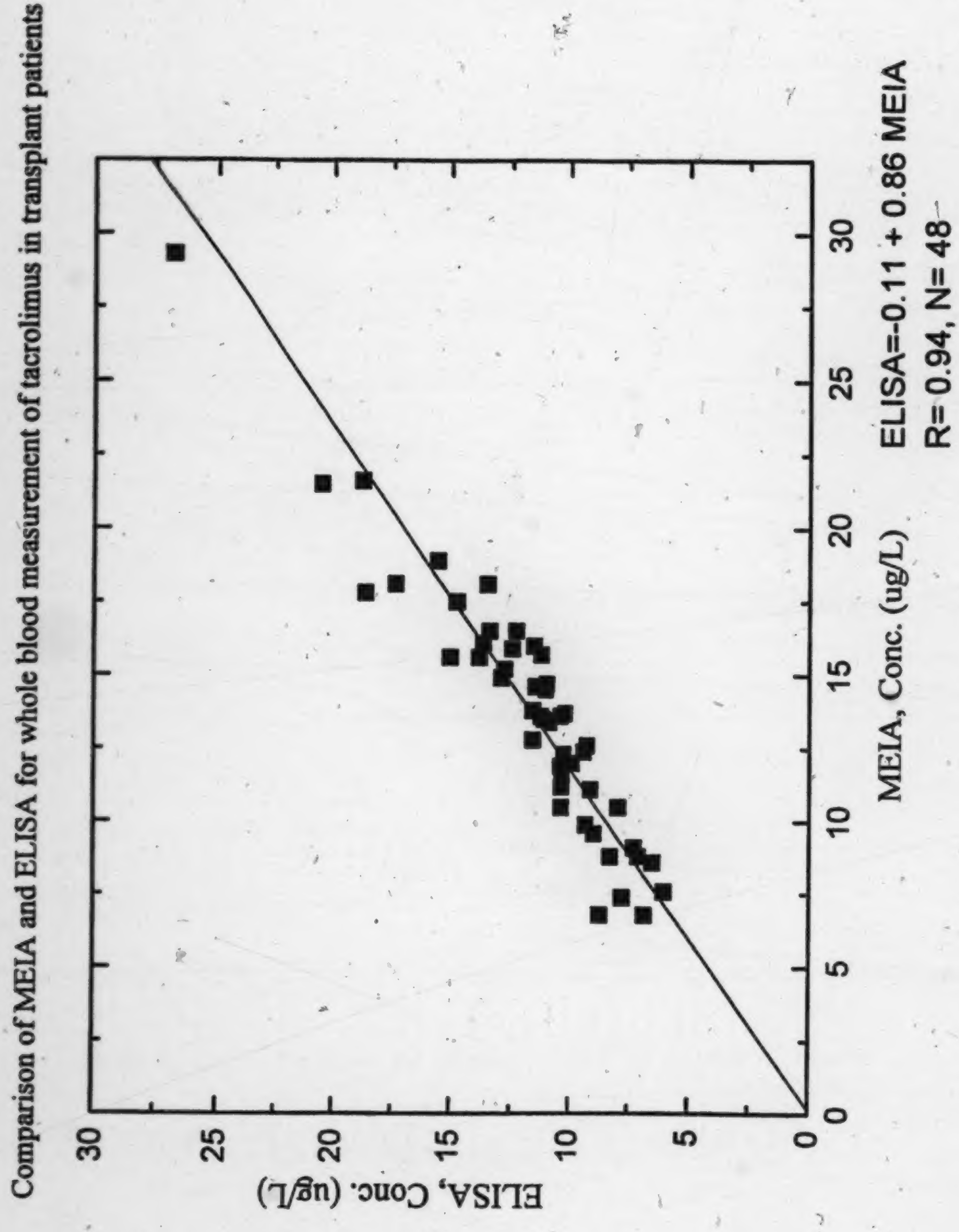


Figure III- 4: The linear regression for the results of FK506 trough blood concentration measured between MEIA and ELISA

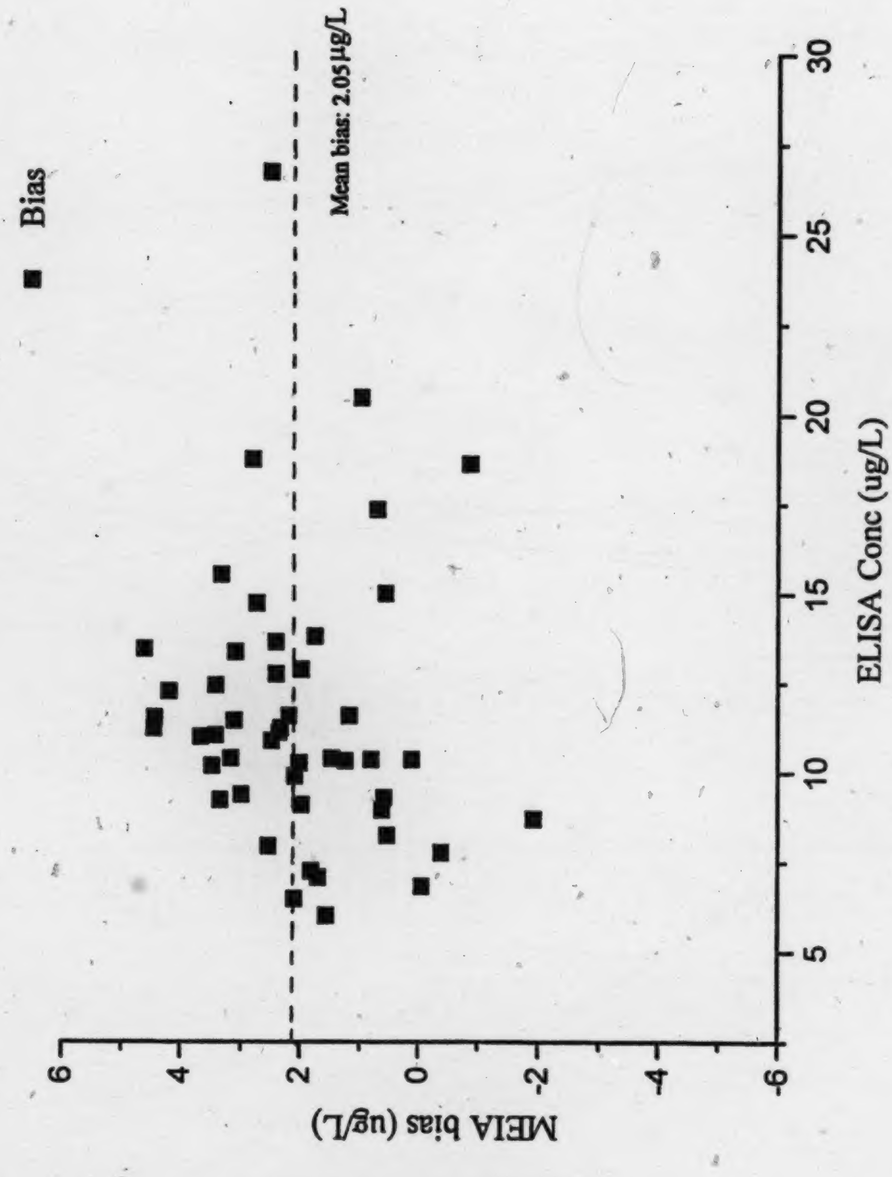


Figure III- 5: Bias plot between MEIA and ELISA.

# Chapter IV

## Biological significance of FK506 metabolites

### Objective

Although FK506 has been used as an immunosuppressive drug for about seven years (Shaw et al., 1999), the biological influence of FK506 metabolites on the efficacy of the drug, the therapeutic monitoring of the parent drug, and side effects of the drug are not fully understood. The impact of FK506 metabolites is dependent on several factors: the steady state concentration, the immunosuppressive ability, the cross-reactivity with antibody used in therapeutic monitoring, and the toxicity. Different assays were used in order to investigate these properties of FK506 metabolites. LC/MS was used to measure the steady state concentration of each metabolite in trough patients' blood. Both the mixed lymphocyte reaction and the calcineurin (CN) inhibitory assay were used to evaluate the immunosuppressive ability of each metabolite. Renal toxicity is the major side effect of FK506. There was evidence to suggest that the pathogenesis of FK506's renal side effect involved alterations in the release of vasoconstrictive substances and/or vasodilatory prostanoids from the vaso-endothelium cells (Brenner et al., 1986; Copeland et al., 1992; Scharschmidt et al., 1983). The effect of FK506 and its metabolites on the release of prostacyclin (prostaglandin $I_2$, $PGI_2$) and on endothelin-1 (ET-1) production in primary cultures of pig aorta endothelium cells was used to investigate the possible mechanism of renal toxicity of FK506 and its metabolites. The objective of these experiments was to investigate the biological significance of FK506 metabolites in order to make a judgement of the possible roles of the FK506 metabolites in the therapeutic and toxic effects of the parent drug.

## Materials and Methods

### LC/ MS assay to measure the steady state concentrations of FK506 metabolites in patients' trough blood

Materials

FK506 standards- in-house prepared by spiking 10 μg/L FK506 stock in normal human blood

Acetyl-FK506 (in-house internal standard)

Liquid chromatography/Mass spectrometry (LC/MS) system- Series 1100, Hewlett Packard, Canada.

$C_{18}$ High performance liquid chromatography (HPLC) column- Nova-Pak, 3.9×150 mm, Waters, USA.

Diethylether- Sigma- Aldrich, Canada. HPLC grade

Acetic acid- Caledon Inc. Canada.

Methanol- Sigma - Aldrich, Canada. HPLC grade

Acetonitrile- Sigma- Aldrich, Canada. HPLC grade

Bench top centrifuge- IEC Centra GP8, Fisher, Canada.

Vortex- Fisher Scientific, Canada.

Rocker- Fisher Scientific, Canada.

Method

The preparation of FK506 standards was previously described in chapter III. An LC/MS system was developed as a reference method to monitor FK506 and possible metabolites. Two tandem $C_{18}$ HPLC columns heated at 50°C were used for HPLC separation. The target solution (FK506 standard or test solution) was first injected into the HPLC system and separated by using a methanol (MeOH)/acetonitrile (ACN)/acetic water (pH 3) gradient at a flow rate of 1.25 mL/min. The gradient profile was as follows: 0-15 minutes: 27% MeOH/30% ACN/43% pH 3 water → at 25 minutes: 40% MeOH/30% ACN/30% pH 3 water → at 30 minutes: 60% MeOH/30% ACN/10% pH 3 water → 35 minutes: 100% MeOH. The HPLC column effluent was then introduced into the mass spectrometer via an electrospray interface. The instrument was operated in selected ion mode. The masses of the ions were chosen depending on whether FK506 alone or together with metabolites needed to be monitored. The mass of FK506 ion was 826 with sodium add-up. The masses of other metabolites were calculated according to possible metabolism pathways such as demethylation (812), hydroxylation (842), didemethylation (798), demethylation-hydroxylation (828), didemethylation-hydroxylation (814), and didemethylation-trihydroxylation (846). When identifying FK506 metabolites, only the correct masses that did not appear in the LC/MS profile of a control drug-free blank extract were recorded. The concentration of each metabolite was measured according to a standard curve of FK506, assuming that each metabolite has the same extraction recovery rate as the one of FK506. This assumption was based on the similarity of the chemical structure and the comparable hydrophobicity between FK506 and its metabolites. The quality controls (LQC, MQC, and HQC) were assessed the same as previously described (chapter III).

### Two-way mixed lymphocyte reaction

#### Materials

Sodium Heparin blood collection tubes – Vacutainer, Canada.

Two HLA- incompatible human subjects

RPMI-1640 media– Gibco laboratories, USA.

Sodium bicarbonate- Fisher, Canada.

Heat-deactivated human type O serum-0.45 μm filtered, volunteers from blood center, Edmonton, Canada.

Histopaque- Pharmacia, USA.

Penicillin/streptomycin- Gibco laboratories, USA

500 mL sterile 45 μm filter with bottle- Fisher, Canada.

Sterile 96-well U shape micro-titer plates- Falcon, USA.

Hemacytometer- Fisher, Canada.

Microscope- Leica Galen III, VWR Scientific, Canada.

Sterile 1-mL, 5-mL, and 10-mL pipette, VWR Scientific, Canada.

Sterile normal saline- Sigma-Aldrich, Canada.

0.02 % (v/v) Trypan blue

$^3$H-Thymidine- Pharmacia, USA.

Glass microfibre filters GF/A- Whatman, USA.

Cell harvesting vacuum bucket

37 °C incubator (humidified atmosphere with 5% $CO_2$)- VWR Scientific, Canada.

Temperature-controlled centrifuge- Centaur 2 MSE, Johns Scientific Inc. USA.

Plexiglass shield – VWR Scientific, Canada.

Blue absorbent pads - VWR Scientific, Canada.

Scintillation fluid – Fisher, Canada.

Scintillation vials – Minivial, USA.

β - Counter – Taurus automatic liquid scintillation counter

Inspector radiation monitor – VWR Scientific, Canada.

Bench top centrifuge- IEC Centra GP8, Fisher, Canada.

### Method

Blood from two HLA- incompatible donors was collected in heparinized tubes via venipuncture. 15 mL was enough for 1 ½ to 2 sterile 96-well plates. The following procedures were conducted using sterile technique in a laminar flow hood. The blood from each donor was diluted 1:1 with sterile saline. 3 parts of diluted blood was layered on 1 part histopaque in a 50 mL blue max tube. The layers needed to be placed carefully without any disturbance. The blue max tubes were centrifuged for 30 minutes at 1000 ×g. The lymphocyte layer was removed into a new blue max tube and topped up with saline to a total volume of 40 mL. The tubes were centrifuged for 10 minutes at 1200 ×g. The supernatant was decanted and the pellet was washed again with 15 mL saline by centrifuging for 10 minutes at 1200 ×g. The washing step was repeated twice. The final pellet was re-suspended in 5 mL RPMI-1640 complete medium. 100 μL of the re-suspended solution was drawn out and added to 100 μL of 0.2% trypan blue. A drop of the mixture was applied to the hemacytometer. The lymphocytes were counted as follows: The numbers (A) of the lymphocytes were counted twice and averaged on the hemacytometer under the microscope. Dead cells were stained blue. The cell concentrations of the mixture from both donors were then adjusted to $1 \times 10^6$ cells / mL according to the equation:

$$\frac{A \times 2 \times 5 \times 10^4 \times 5 \times}{1 \times 10^6} \tfrac{1}{2} = \text{Total volume of RPMI-1640 complete medium}$$

To each well of the sterile 96-well plate, 100 μL of each donor's cells plus 20 μL of an appropriate concentration of FK506, metabolites, or media control in RPMI-1640 were added; the assays were done in quadruplicate. The plates were incubated for 5 days at 37 °C in a 5% $CO_2$ incubator. On the afternoon of day 5, 30 μL of 1 μCi $^3$H-thymidine was added to every well. The plates were incubated for another 18 hours and the cells were harvested at the end of incubation using filter paper. Each filter paper was dried in a scintillation vial for at least 6 hours in a well-ventilated area. 2.5 mL of scintillation fluid was added to each vial after the filter paper was dried. The vials were capped and shaken, and the radioactivity was counted on β-counter. Results were expressed as % inhibition calculated as follows: [1-(cpm of drug/cpm of zero control)] ×100. Zero control was the reaction without any drug. The $IC_{50}$ values for FK506 and its metabolites were calculated according to the successive results from various concentrations of each target.

### Cross- reactivity study

### Materials

Pro-Trac II FK506 Enzyme-Linked-Immunosorbent-assay (ELISA) kits- INCSTAR Co. USA

Purified FK506 metabolites stock (16 species) in various concentrations

12 × 75 mm conical bottom tubes- VWR Scientific, Canada.

Double deionized water

Blood rotator- Fisher, Canada.

Tube shaker- Fisher, Canada.

Water bath- VWR Scientific, Canada.

Plate shaker- VWR Scientific, Canada.

Timer- Fisher, Canada.

Microplate spectrometer- VWR Scientific, Canada.

Ice bucket- VWR Scientific, Canada.

Ice machine- VWR Scientific, Canada.

Bench top centrifuge- IEC Centra GP8, Fisher, Canada.

Micro pipette (1-10 μL, 10-50 μL, 100-1000 μL)- Nichipet, Canada.

Micro pipette tips- VWR Scientific, Canada.

## Methods

An ELISA assay for monitoring FK506 was used to investigate the cross-reactivity of FK506 metabolites and was previously described in chapter II. The spiked final concentrations of the metabolites in human normal blood are as follows: FK506-10 μg/L, 13-demethyl FK506-38 μg/L, 15-demethyl FK506-14.75 μg/L, 31-demethyl FK506-9.36 μg/L, 13,15-didemethyl FK506-35 μg/L, 13,31-didemethyl FK506-39 μg/L, 15,31-didemethyl FK506- 54 μg/L, demethyl-hydroxy (1)-22.5 μg/L, demethyl-hydroxy (2)-43.2 μg/L, demethyl-hydroxy (3)-61.5 μg/L, demethyl-hydroxy (4)-34.2 μg/L, demethyl-hydroxy (5)-52.25 μg/L, didemethyl-hydroxy-44 μg/L, didemethyl-trihydroxy-48 μg/L, hydroxy (1)-46 μg/L, hydroxy (2)-32 μg/L, and hydroxy (3)-43 μg/L. Each metabolite was tested three times. The determination of various concentrations of FK506 metabolites used in the assay was according to preliminary tests (data not shown). The cross-reactivity of each FK506 metabolite was assessed by comparing the actual concentration of the metabolite measured from ELISA and the theoretical concentration of the metabolite. The results are presented as percentage cross-reactivity.

## Calcineurin (CN) inhibitory assay

### Materials

Plexiglass shield – VWR Scientific, Canada.

Specially made Plexi glass column holders with matching vial racks- VWR Scientific, Canada.

Blue absorbent pads - VWR Scientific, Canada.

Scintillation fluid – Fisher, Canada.

Scintillation vials – Minivial, USA.

$\beta$ - Counter – Taurus automatic liquid scintillation counter

Inspector radiation monitor – VWR Scientific, Canada.

19-amino acid peptide as target of calcineurin, produced by the protein synthesis group at University of Alberta.

Okadaic acid – Calbiochem, USA.

Catalytic subunit of cAMP-dependent protein kinase- Sigma- Aldrich, Canada.

Leupeptin – Calbiochem, USA.

Aprotinin – Calbiochem, USA.

Ethylenediaminetetraacetic acid (EDTA) – Calbiochem, USA.

Ethylene glycol-bis ($\beta$-aminoethyl ether) (EGTA) – Calbiochem, USA.

$\beta$-Mercaptoethanol (BME) – Sigma- Aldrich, Canada.

Tween-80 - Sigma- Aldrich, Canada.

[$\gamma$-$^{32}$P] ATP- Pharmacia, USA.

ATP- Sigma- Aldrich, Canada.

MES – Sigma- Aldrich, Canada.

Protein kinase A – Boehringer Mannheim, USA.

C18 extraction cartridge – Waters, USA.

Dowex 50WX8-400 – Lancaster, Canada.

Tris base – Calbiochem, USA.

Trichloroacetic acid – Lancaster, Canada.

NaOH – Caledon, Canada.

Potassium phosphate – Mallinckrodt, USA.

Dithiothreitol (DTT) – Lancaster, Canada.

Phenylmethylsulfonyl fluoride (PMSF) – Calbiochem, USA.

Soybean trypsin inhibitor – Sigma- Aldrich, Canada.

NaCl – Lancaster, Canada.

HCl – Caledon, Canada.

$MgCl_2$ – Calbiochem, USA.

$CaCl_2$ – Lancaster, Canada.

Bovine serum albumin – Sigma- Aldrich, Canada.

Calibrated Eppendorf Pipette (1.0 mL, 200 μL, and 0.5-10 μL)

Vortex – Fisher, Canada.

Rocker – Fisher, Canada.

Disposable tubes with caps – Falcon, USA.

Liquid nitrogen – Praxair, Canada.

Sodium heparin blood collection tubes – Vacutainer, Canada.

Magnetic stirrer – Fisher, Canada.

Water bath – Fisher, Canada.

Timer – VWR Scientific, Canada.

Ice bucket – Fisher, Canada.

Ice Machine – VWR Scientific, Canada.

Calibrated thermometer, VWR Scientific, Canada.

15 and 50 mL conical tubes – VWR Scientific, Canada.

Styrofoam container

Gibco bottles (500 mL) and caps – Fisher, Canada.

100 and 500 mL graduated cylinder – VWR Scientific, Canada.

## Methods

The method described below was adapted from a similar method published in several papers (Batiuk et al., 1996; Quien et al., 1997; Halloran et al., 1999). The differences were: 1) The concentration of β- mercaptoethanol (BME) in lysis buffer was 0.01 % instead of 0.1 %. 2) The saturating dose of FK506 was added to a portion of the reaction mixture 30 minutes before lysis took place in order for equilibration to occur. In order to investigate the influence of FK506 and its metabolites on the activity of calcineurin (CN), the experiment was conducted using three groups. The first group (control) was used to measure the enzyme activity of normal fresh whole blood; the second group (A) was used to measure the enzyme activities of freshly collected blood that spiked with various concentrations of FK506 or its metabolites. The third group (B) (containing another set of control and A) was saturated by 300 μg/L FK506. All groups were incubated at 37 °C for 30 minutes before lysis. Whole blood collected into sodium heparin tubes was the source of CN. Blood had to be kept at room temperature after collection and lysed within 24-48 hours; otherwise, the enzyme would be labile, possibly invalidating the results. Each sample was lysed into three aliquots, the volume of each aliquot being 190 μL. These aliquots of the lysed samples were stored at – 70 °C until analysis. Samples had to be analyzed within 3 weeks. The hypotonic lysis buffer consisted of 50 mM Tris pH 7.5, 1 mM DTT, 50 μg/mL PMSF, 50 μg/mL soybean Trypsin inhibitor, 10 μg/mL Leupeptin, 10 μg/mL Aprotinin, 1 mM EDTA pH 8.0, 100 μM EGTA pH 7.0, 0.01 % BME, and deionized water. PMSF was freshly prepared in 95% ethanol as a stock solution of 10 mg/mL. 150 μL whole blood and 40 μL lysis buffer were mixed well in micro-centrifuge tubes and frozen in liquid nitrogen for a few minutes. Once 3 aliquots had been lysed, the original samples could be discarded.

A peptide consisting of 19 amino acids was synthesized by the protein synthesis group at the University of Alberta. The sequence was as follows: Aspartic acid (D), Leucine (L), Aspartic acid, Valine (V), Proline (P), Isoleucine (I), Proline, Glycine (G), Arginine (R), Phenylalanine (F), Aspartic acid, Arginine, Arginine, Valine, Serine (S), Valine, Alanine, Alanine, Glutamic acid (E) (Blumenthal, 1986). The major function of CN was to dephosphorylate the serine residue of this peptide, so the peptide was labeled with Protein kinase A by [γ-$^{32}$P] ATP at the serine site to a specific activity of 90 mCi/mmol. Briefly, protein kinase A was added in the peptide phosphorylation buffer that contained 300 μM ATP, 148.5 μM peptide, 20 mM MES, 200 μM EGTA, 400 μM EDTA, 2 mM $MgCl_2$, 50 μM, 50 μg/mL BSA, 10 mCi/mL [γ-$^{32}$P] ATP and deionized water to initiate the labeling reaction. The mixture was incubated at 30 °C for 60 minutes. The reaction solution was left for 10 minutes at room temperature. A C18 extraction cartridge was washed with 3 mL ACN (30% acetonitrile in 0.1 % trifluoroacetic acid) and 5 mL TFA (0.1% trifluoroacetic acid) during the reaction. At the end of the reaction, each 1 mL of peptide reaction solution was added to a C18 extraction cartridge. Once the peptide solution entered the cartridge bed, 20 mL TFA was added to wash the cartridge. The peptide was then eluted into a new collection tube using 3 mL of ACN. The first 0.5 mL of ACN was discarded. The solvent in the peptide eluate was evaporated using a stream of air over-night. The dried peptide residue was dissolved in 0.5 mL of final peptide buffer that contained 20 mM Tris, pH 8.0, 100 mM NaCl, 100 mM $MgCl_2$, 100 μM $CaCl_2$, 500 μM DTT, 100 μg/mL BSA and deionized water. The activity of CN was then analyzed by measuring the release of free $^{32}$P that was collected using an ion exchange cartridge, Dowex (50WX8–400).

Dowex (50WX8–400) was pre-treated before putting it into the cartridges. Briefly, 250 g of Dowex was mixed with 600 mL de-ionized water in a large beaker for 15 minutes. The water was discarded after 15 minutes of the resin settling down. This washing procedure with water was repeated four more times. Then, 600 mL of 1 N NaOH was mixed with the Dowex for 1 hour. NaOH was poured off after 15 minutes of the resin settling down. 600 mL of HCl was added to the Dowex and the mixture was stirred for 1 hour. The HCl was poured off after 15 minutes of the resin settling down. The mixing with HCl was repeated once more. Then, the Dowex was washed with de-ionized water four times. The final Dowex was in 600 mL of fresh de-ionized water for the CN assay.

The CN assay indirectly measured the % CN activity or % CN inhibition. There are other phosphatases, e.g. PP1, PP2a and PP2c, that would also dephosphorylate the peptide. Okadaic acid was used in the assay in order to inhibit the activities of PP1 and PP2a. A saturating dose of FK506 was added to a portion of the reaction. The total CN activity can be calculated by subtracting the radioactivity of the release of free $^{32}$P from samples without FK506 saturation from the ones with FK506 saturation (control or group A minus group B). The basic assay buffer consisted of 20 mM Tris pH 8.0, 100 mM NaCl, 6 mM $MgCl_2$, 500 μM DTT, 100 μg/mL BSA, 750 nM okadaic acid, 100 μM $CaCl_2$, 15 μM labeled peptide, and de-ionized water. The peptide was not added until just before the reaction took place.

The procedure for the CN assay was as follows: Duplicate lysed samples (control, A& B) were frozen and thawed three times between liquid nitrogen and lukewarm water. The thawed samples were centrifuged for 10 minutes at 12000 ×g. 30 μL of each sample were added to three sets of reaction tubes (control, A & B). 30 μL of water was also added to three groups (control, A& B) as a measure of cell-free reaction. The ion exchange cartridges were prepared at this time by filling the cartridges with stirred pre-treated Dowex. 60 μL of basic assay buffer was added to each group A tube every 15 seconds. The same step was repeated for basic assay buffer with group B tubes. The reaction solution was mixed well and placed in the 30 °C water bath for 15 minutes. 500 μL of ice-cold stop solution was added to stop the reaction after 15 minutes of incubation. The stop solution was made up of 50 mL of 1 mol/L potassium phosphate and 25 g trichloroacetic acid. The stopped reaction solution was poured into the cartridge followed by 500 μL of water, and then a further 400 μL of water. All solutions (1650 μL) were collected into scintillation vials. 5 mL of scintillation fluid was added to each scintillation vial. The vials were capped and vortexed well. The positive control (basic assay buffer), cell-free controls, and samples were counted on the β counter for one minute to detect the radioactivity of $^{32}P$. The percentage of inhibition of CN for each sample was calculated. The $IC_{50}$ values for CN inhibition for each FK506 metabolite were also calculated and reported in the results.

### Culture of pig aorta endothelial cells

#### Materials

Pig aorta endothelium cells- Cell systems, USA.

M-199 culture medium- Gibco laboratories, USA.

Fetal bovine serum- Gibco laboratories, USA.

Trypsin-EDTA- Gibco laboratories, USA.

Sterile 96-well flat bottom micro-titer plates- Falcon, USA.

$^{3}H$-Thymidine- Pharmacia, USA.

37 °C incubator (humidified atmosphere with 5% $CO_2$)- VWR Scientific, Canada.

Temperature-controlled centrifuge- Centaur 2 MSE, Johns Scientific Inc. USA.

Plexiglass shield – VWR Scientific, Canada.

Blue absorbent pads - VWR Scientific, Canada.

Scintillation fluid – Fisher, Canada.

Scintillation vials – Minivial, Canada.

β - Counter – Taurus automatic liquid scintillation counter

Inspector radiation monitor – VWR Scientific, Canada.

Sterile culture flasks- VWR Scientific, Canada.

Microcentrifuge tubes- Sorenson BioScience Inc. USA.

Micro pipette (1-10 µL, 10-100 µL, 100-1000 µL)- Nichipet, Canada.

Micro Pipette tips- DiaMed Lab Supplies Inc, Canada.

## Method

The following procedures were done using sterile techniques. They took place in a sterile biohazard hood, and the equipment used in the procedure was sterile. The primary pig aorta endothelial cells were transfered from frozen vials into sterile flasks. The growth medium used was M-199 with 20% (v/v) fetal bovine serum and 5% (v/v) Penicillin/Streptomycin. Microscopic examination of the cultures was done after one week of incubation to ensure a homogenous population of polygonal cells. The cells were then passed in another sterile flask with M-199 medium changed to 10 % (v/v) fetal bovine serum, and in sterile 96-well flat bottom plates with medium changed to 10 % (v/v) fetal bovine serum and containing no Penicillin/Streptomycin. The above step was done repeatedly in order to have the second, third, and fourth generations of the cell culture ready for the experiments. Following the passage of the cells to the 96-well plates, the cells were incubated at 37 °C for another three days until confluence. The mono-layered cells were checked under microscopy to ensure their viability. The cells were washed gently once by filling the wells with M-199 without any addition of serum and antibiotic. This type of medium was used when conducting the experiments. The washing medium was withdrawn from the wells and the new medium with various concentrations of FK506 and its metabolites was poured into the wells. The experiments included quadruplicate wells for each concentration and positive and negative controls. The supernatant of each well was harvested after 24 hours of culture. The optimum time for the culture had been determined previously by colleagues in the laboratory. Half of the supernatant from each concentration and control was for the measurement of endothelin-1,2 by radioimmunoassay. The other half was for the measurement of 6-keto-prostaglandin $F_{1\alpha}$, a primary metabolite of prostacyclin, by radioimmunoassay. After the harvest of supernatant, fresh M-199 medium containing 1 μCi $^3$H-thymidine was applied into the wells. 10 μL of trypsin-EDTA was added to each well and left for 5 minutes at 37 °C to lift the cells from the bottom of plate after 8 hours of culture. The medium together with the cells was harvested onto filter paper. The filter paper was dried for 8 hours in the scintillation vials. The radioactivity for each sample was counted in a β counter. These results represented the cell growth of each well, and the results from the measurements of endothelin 1,2 and 6-keto-prostaglandin $F_{1\alpha}$ (metabolite of prostacyclin) were normalized by the growth rate of the cells (Copeland et al., 1990; Copeland et al., 1992; Langman et al., 1994).

$^{125}$I radioimmunoassays for endothelin 1,2 and 6-keto-prostaglandin $F_{1\alpha}$

Materials

Endothelin 1,2 [$^{125}$I] assay system with magnetic separation- Amersham, USA.

6-Keto-prostaglandin $F_{1\alpha}$ [$^{125}$I] (primary metabolite of prostacyclin) assay system with magnetic separation- Amersham, USA.

Plexiglass shield – VWR Scientific, Canada.

Plexiglass container – VWR Scientific, Canada.

Ventilated fume hood - VWR Scientific, Canada.

Blue absorbent pads - VWR Scientific, Canada.

Scintillation fluid – Fisher, Canada.

Scintillation vials – Minivial, USA.

β - Counter – Taurus automatic liquid scintillation counter

Inspector radiation monitor – VWR Scientific, Canada.

Pipettes- Nichipet, Canada.

Disposable pipette tips- DiaMed Lab Supplies Inc, Canada.

Refrigerator- 4 °C, Fisher, Canada.

Vortex- Fisher, Canada.

Timer- Fisher, Canada.

Disposable polypropylene tubes (12 × 75mm)- VWR Scientific, Canada.

Glass measuring cylinder (100 mL)- VWR Scientific, Canada.

Deionized water

Microcentrifuge tubes- Sorenson BioScience Inc. USA

Refrigerated bench top microfuge tube centrifuge, VWR Scientific, Canada.

Decantation racks- VWR Scientific, Canada.

Microcentrifuge tube racks- Sorenson BioScience Inc. USA

## Method

### A. Endothelin 1,2 [$^{125}$I] assay

All reagents and samples were equilibrated to room temperature. The assay buffer was diluted using deionized water according to the procedure provided by the company (Amersham, USA). Standards, antiserum, and tracer were also diluted as required for the procedure using the working assay buffer. The polystyrene tubes (in duplicate) for total counts (TC), non-specific binding (NSB), zero standard ($B_0$), standards, and samples were labeled. 200 μL of assay buffer was pipetted into NSB tubes and 100 μL into $B_0$ tubes. 100 μL of each standard or sample (supernatant from the culture of pig aorta endothelium cells) was pipetted into an appropriate tube. 100 μL of antiserum was then pipetted into all tubes except those for NSB and TC. All tubes were mixed thoroughly and covered with plastic film. The tubes were incubated for 4 hours at 2-8 °C. After the incubation, 100 μL of [$^{125}$I] ET-1 solution was pipetted into all tubes. The TC tubes were stoppered and these tubes were put aside for counting. The rest of the tubes were mixed well, covered, and incubated for 16-24 hours at 2-8 °C. At the end of reaction, 250 μL of Amerlex-M second antibody reagent (blue-green) was added to all tubes except the TC tubes. The tubes were incubated at room temperature for 10 minutes then centrifuged at 4 °C for 10 minutes at 1500 ×g or greater. The tubes were put carefully into suitable decantation racks and then the supernatant was poured off and discarded. The tubes were kept inverted and placed on a pad of absorbent tissues for draining for about 5 minutes. The rims of the inverted tubes were firmly blotted on the tissue pad to remove any adhering liquid. The tubes cannot be re-inverted once they have been turned upright because the pellet in the bottom of the tubes maybe disturbed by the left-over liquid and is easily lost if re-inverted. The radioactivity present in each tube was determined by counting for at least 1 minute in a β - scintillation counter. The data collected were calculated according to the rules described in the menu for the procedure. A standard curve was generated by plotting the percent $B/B_0$ of the standards as a function of the log ET-1 concentrations (fmol).

$$\%B/B_0 = \frac{\text{(Standard or sample cpm- NSB cpm)}}{B_0 \text{ cpm- NSB cpm}} \times 100$$

The fmol per test tube can be read directly from the graph.

## B. 6-Keto-prostaglandin $F_{1\alpha}$ [$^{125}$I] assay

6-Keto-prostaglandin $F_{1\alpha}$ is the primary metabolite of prostacyclin. All reagents were equilibrated to room temperature and diluted immediately prior to performing the assay. The polypropylene tubes (12 × 75 mm) were labeled in duplicate for total count (TC) tubes, non-specific binding (NSB) tubes, zero standard ($B_0$) tubes, standards and samples. 300 µL of assay buffer was pipetted into the $B_0$ tube and 400 µL into the NSB tubes. 200 µL of assay buffer was pipetted into all standard and sample tubes. 100 µL of each standard or sample was pipetted, in duplicate, into the appropriately labeled tubes and mixed well. [$^{125}$I] 6-Keto-prostaglandin $F_{1\alpha}$ (100 µL) was pipetted into all tubes and mixed well. 100 µL of antiserum was pipetted into all tubes except the TC tubes and NSB tubes. The TC tubes were stoppered and these tubes were put aside for counting. All other tubes were mixed thoroughly for 2-5 seconds and incubated overnight (between 15 and 18 hours) at 2-8 °C. At the end of incubation, 500 µL Amerlex-M second antibody reagent (blue-green) was added to all tubes except the TC tubes. All tubes were mixed thoroughly and incubated for 10 minutes at room temperature and then centrifuged for 10 minutes at 1500 ×g or greater. The tubes were put carefully into suitable decantation racks and then the supernatant was poured off and discarded. The tubes were kept inverted and placed on a pad of absorbent tissues for draining about 5 minutes. The rims of the inverted tubes were firmly blotted on the tissue pad to remove any adhering liquid. The tubes cannot be re-inverted once they have been turned upright because the pellet in the bottom of the tubes maybe disturbed by the left-over liquid and is easily lost if re-inverted. The radioactivity present in each tube was determined by counting for at least 1 minute in a β-scintillation counter. The data collected were calculated according to the rules described in the menu of the procedure. A standard curve was generated by plotting the percent bound as a function of the $\log_{10}$ 6-keto-prostaglandin $F_{1\alpha}$ concentration (pg).

$$\%B/B_0 = \frac{\text{(Standard or sample cpm- NSB cpm)}}{B_0 \text{ cpm- NSB cpm}} \times 100$$

The pg per tube of the samples can be read directly from the graph.

## Results

Table IV-1 shows the steady state concentration, mixed lymphocyte reaction, and cross-reactivity of FK506 and its metabolites with anti-FK506 antibody. The steady state concentrations that were measured represented the average trough concentrations of the identifiable metabolites in blood from 20 patients receiving FK506 as part of their immunosuppressive therapy. FK506, 13-demethyl FK506, 15-demethyl FK506, 13,15-didemethyl FK506 and one demethyl-hydroxy species are always identifiable in patients' blood samples. The FK506 metabolite that is in the highest concentration is 13-demethyl FK506. It is approximately 10 fold less than the concentration of FK506. The tests of mixed lymphocyte reaction and cross-reactivity with anti-FK506 were done three times at different dates. 31-demethyl FK506 has a very similar immunosuppressive potency to that of FK506, but it is not identifiable in patients' blood. With the FK506 antibody used in immunoassays, 31-demethyl FK506 has over 100% cross-reactivity. 15-demethyl FK506 and 15,31-didemethyl FK506 have approximately 90% cross-reactivity with anti-FK506.

Table IV-2 shows the results of CN inhibition and the mixed lymphocyte reaction of FK506 and its metabolites. The tests were done three times. 31-Demethyl FK506 caused a similar degree of inhibition in the CN inhibition and mixed lymphocyte inhibition tests. However, 13-demethyl FK506 and 13,31-didemethyl FK506 caused different degrees of inhibition in the two tests. The differences were significant (t- test, $p<0.05$, data not shown).

Figure IV-1 shows the results of the influence of a series of concentrations (1, 2.5, 8.5, 25, 50, 100 μg/L) of FK506 and its metabolites on the release of endothelin 1,2 and prostacyclin from the culture of pig aorta endothelial cells. Figure IV-1 A represents the relative changes of endothelin 1,2 and figure IV-1 B represents the relative changes of prostacyclin. Figure IV-1 C- H shows the influence of FK506, 13-demethyl FK506, 15-demethyl FK506, 31-demethyl FK506, 13,31-didemethyl FK506 and 15,31-didemethyl FK506 on the release of endothelin-1,2 and prostacyclin, and the growth rate of the cell culture. 31-demethylated FK506, 13,31-didemethylated FK506, and 15,31-didemethylated FK506 decreased the growth rate of the endothelial cells at concentrations of 50 and 100 µg/L; FK506 and other metabolites did not have the same effect on the growth rate of the cells. FK506 and 15-demethylated FK506 showed very similar patterns in the concentration-related induction of endothelin. The peak secretion occurred at the free drug concentration of 8.5 µg/L or between 8.5 and 25 µg/L. The other FK506 metabolites did not have any influence on the release of endothelin from pig aorta endothelial cells. 13,31-didemethylated FK506 showed the ability to induce the secretion of prostacyclin, a vaso-dilator, in a inverse dose-related manner (secretion decreases at higher concentration).

| FK506 metabolites | LC/MS retention time (min) | Human blood level (μg/L) | Immuno-suppression (%) | Antibody cross-reactivity (%) |
|---|---|---|---|---|
| *FK506 | 30 | 10.5 ± 3.8 | 100 | 100 |
| *13-demethyl | 11-18:tautomers | 1.6 ± 0.7 | 19.9 ± 6.1 | 0 |
| *15-demethyl | 20.5 | 0.78 ± 0.3 | 0 | 90 ± 2.8 |
| 31-demethyl | 27.5 | - | 98.1 ± 8.2 | >100 |
| *13,15-didemethyl | 6 | - | 0 | 4.6 ± 0.1 |
| *13,31-didemethyl | 7-12:tautomers | 0.34 ± 0.1 | 23.3 ± 4.6 | 3 ± 1.1 |
| 15,31-didemethyl | 14 | - | 7.3 ± 0.6 | 91 ± 1.2 |
| Dm-hydroxy (1) | 3.5 | - | 3 ± 0.35 | 3 ± 0.9 |
| Dm-hydroxy (2) | 4 | - | 1 ± 0.08 | 1 ± 0.05 |
| Dm-hydroxy (3) | 4.5 | - | 13 ± 2.45 | 1 ± 0.08 |
| *Dm-hydroxy (4) | 6 | 0.23 ± 0.06 | 4 ± 0.58 | 2 ± 0.07 |
| Dm-hydroxy (5) | 9 | - | 20 ± 5.5 | 8 ± 1.15 |
| Didm-hydroxy | 3-4 | - | 1 ± 0.7 | 3 ± 0.2 |
| Didm-trihydroxy | 3-4 | - | 1 ± 0.08 | 1 ± 0.04 |
| Hydroxy (1) | 8.5 | - | - | - |
| *Hydroxy (2) | 13.5 | - | 7 ± 1.15 | 18 ± 2.8 |
| *Hydroxy (3) | 21-24 | - | - | - |

*= Exist in human whole blood; Dm= demethyl

Table IV-1: The steady state concentration, immunosuppression, and cross-reactivity with anti-FK506 of FK506 and its metabolites. The steady state concentrations were measured from 20 trough FK506 patients' blood samples. The immunosuppression represents the results from the mixed lymphocyte reaction. The percentage of inhibition for each metabolite was calculated according to the $IC_{50}$ relatively to that of FK506. FK506 ELISA was used for the test of cross-reactivity. The results are expressed as mean ± standard deviation.

| FK506 metabolites | Immusuppression (%) MLR | Calcineurin inhibition (%) (whole blood) | Calcineurin inhibition (%) (PBMC) |
|---|---|---|---|
| FK506 | 100 | 100 | 100 |
| 13-demethyl | 19.9 ± 6.1 | 40.3± 1.9 | 13 ± 0.06 |
| 15-demethyl | 0 | 0.6 ± 0.08 | <0.25 |
| 31-demethyl | 98.1 ± 8.2 | 80.2 ± 5.5 | 100 |
| 13,31-didemethyl | 23.3 ± 4.6 | 5 ± 0.8 | 1.5 ± 0.1 |
| 15,31-didemethyl | 7.3 ± 0.6 | 8.2 ± 0.9 | <0.2 |

Table IV-2: Results of calcineurin inhibition [whole blood vs. peripheral blood mononuclear cells (PBMC)] and the mixed lymphocyte reaction of FK506 and its metabolites. Tests were done three times. All results with FK506 metabolites are presented relative to the results with FK506. The results are expressed as mean ± standard error.

**Figure IV-1**: The results of the influence of FK506 and its metabolites on the realease of endothelin 1,2 and prostacyclin from the culture of Pig Aorta Endothelial cells. *The results represent the findings from three experiments, except in the cases where no error bars are given

*The results are expressed as mean ± standard deviation.

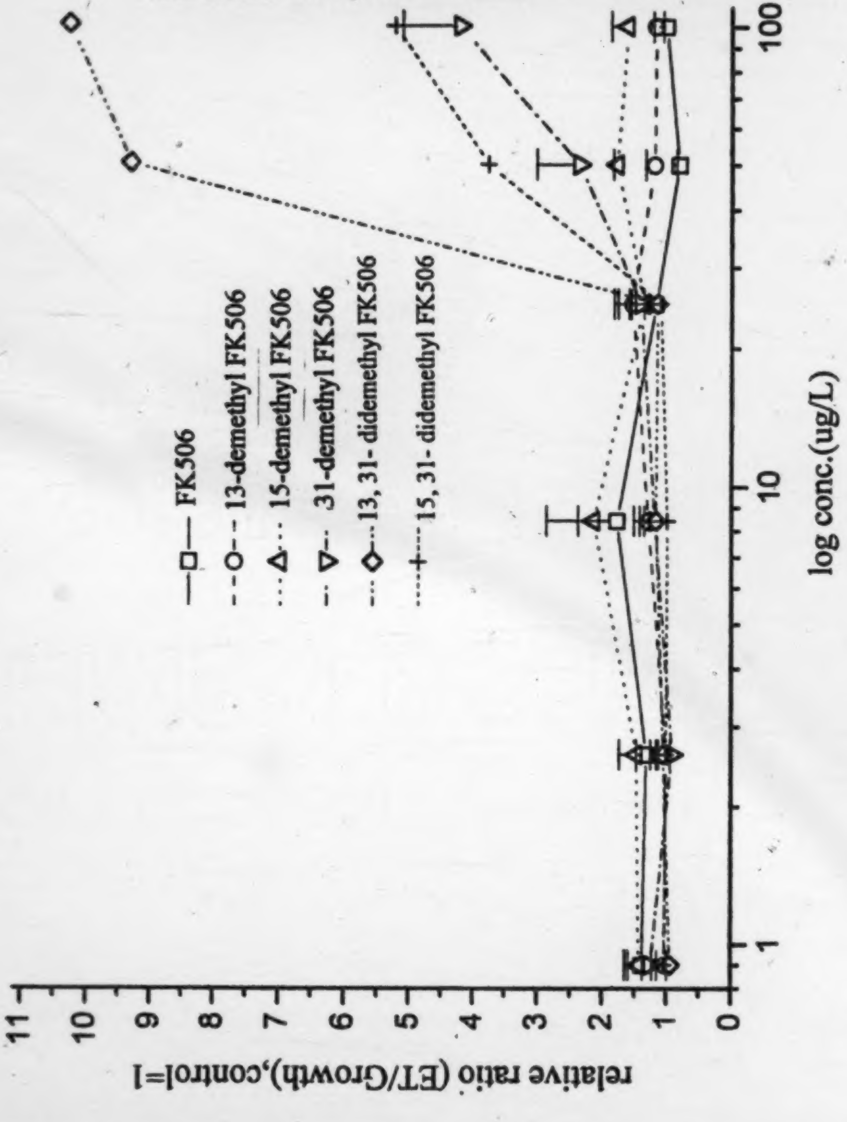


**Figure IV-1 A**: Relative changes of endothelin 1, 2 from the culture of pig aorta endothelial cells.

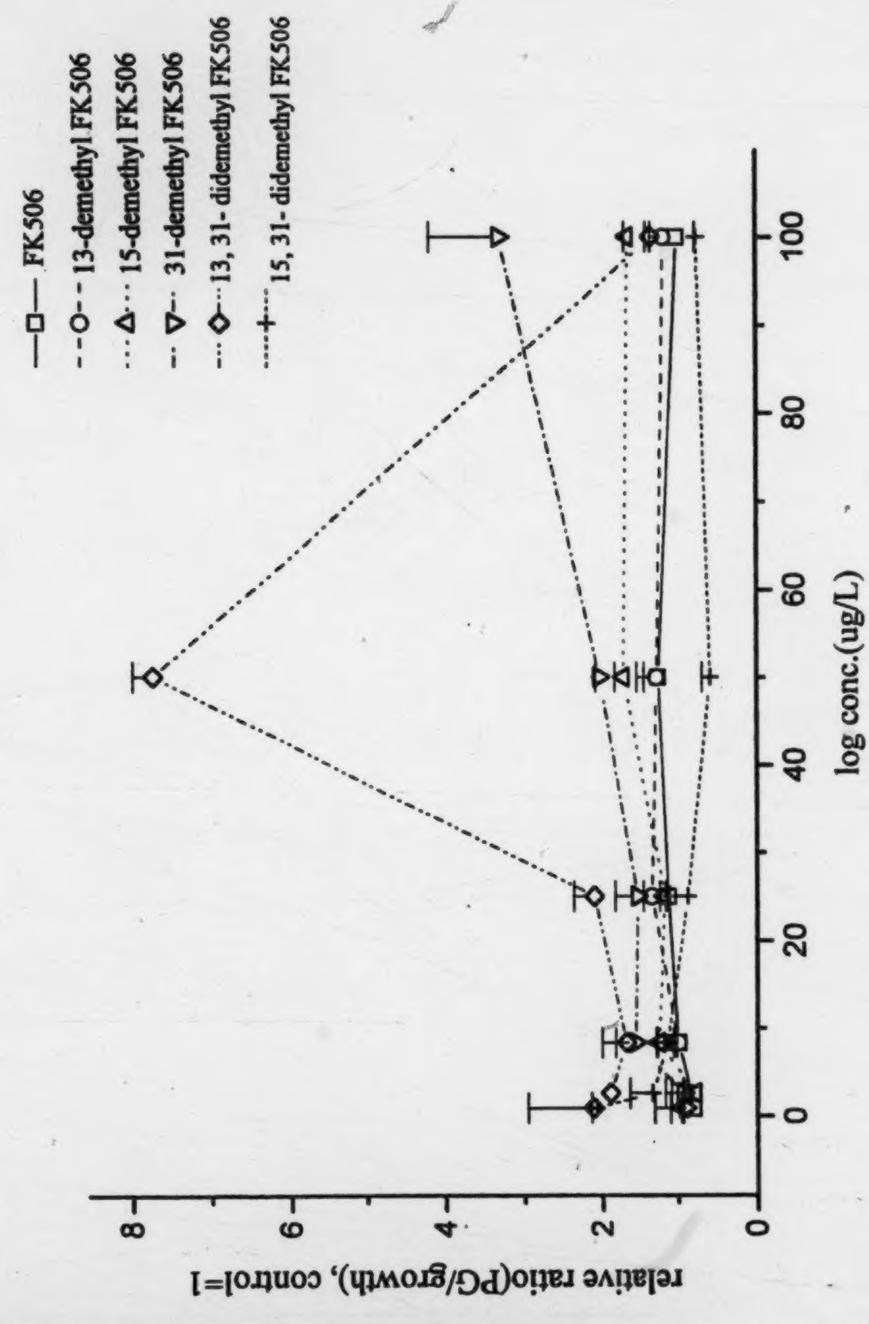


Figure IV-1B: Relative changes of prostaglandin F1α (prostacyclin metabolite) from the culture of pig aorta endothelial cells

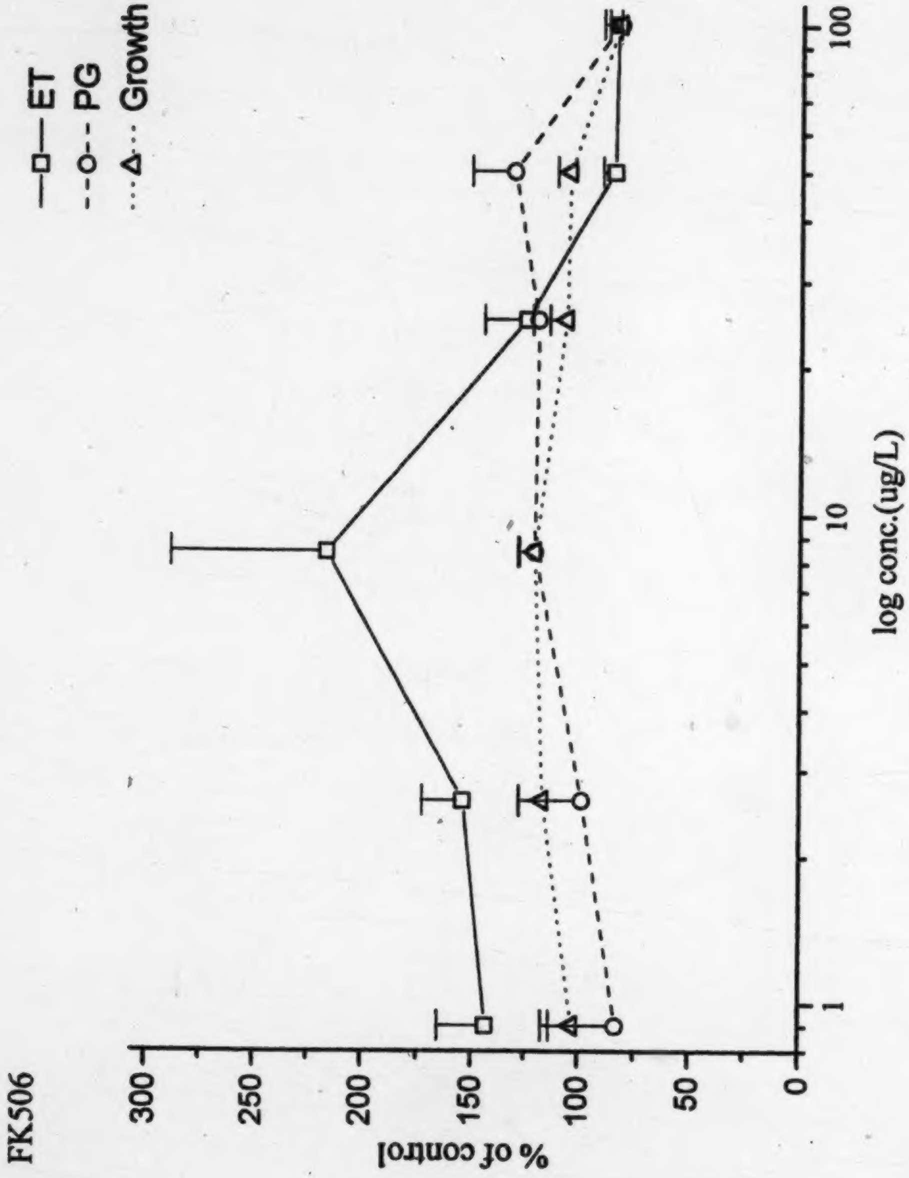


Figure IV-1 C: The influence of FK506 on the release of endothelin- 1, 2 and prostaglandin F1α, and the growth rate of the cell culture.

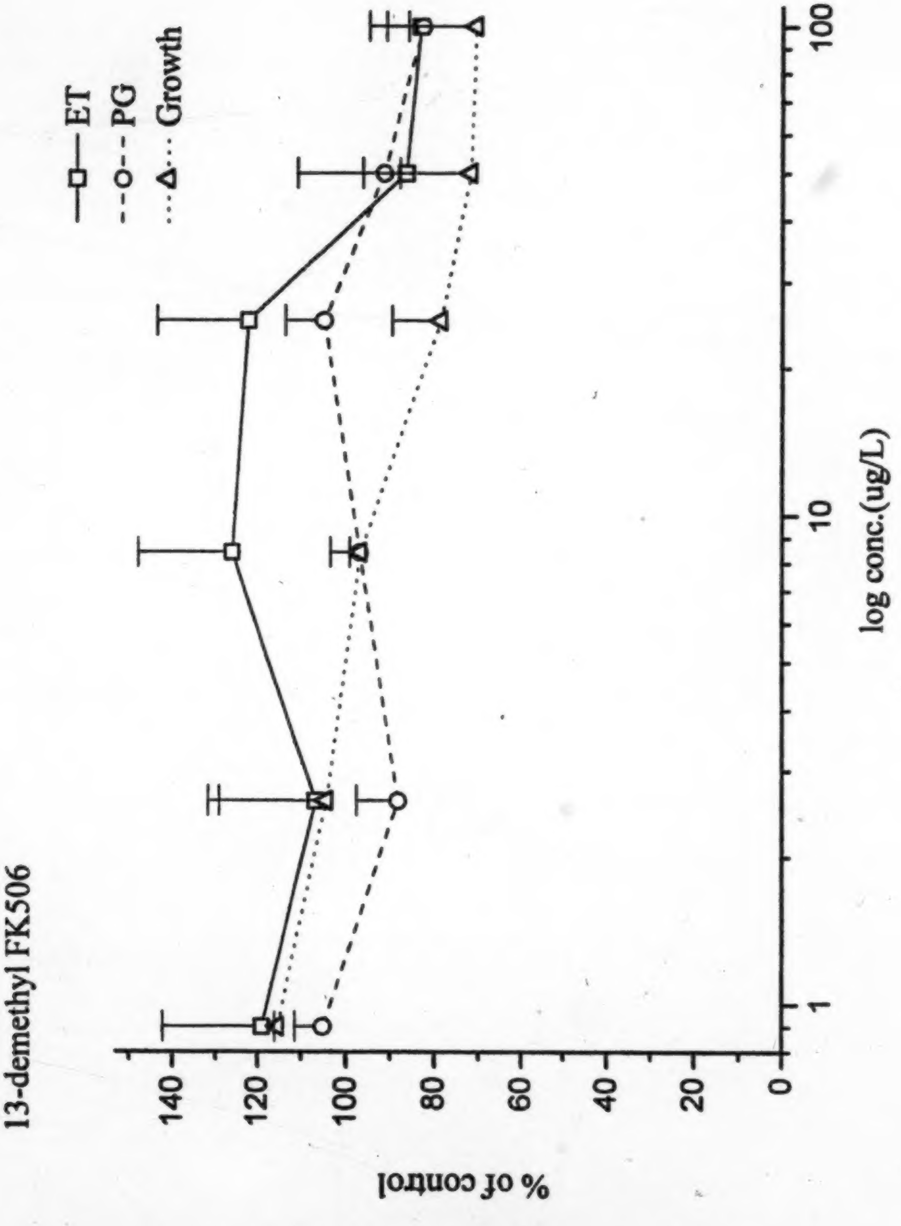


Figure IV-1 D: The influence of 13–demethyl FK506 on the release of endothelin- 1, 2 and prostaglandin F1α, and the growth rate of the cell culture.

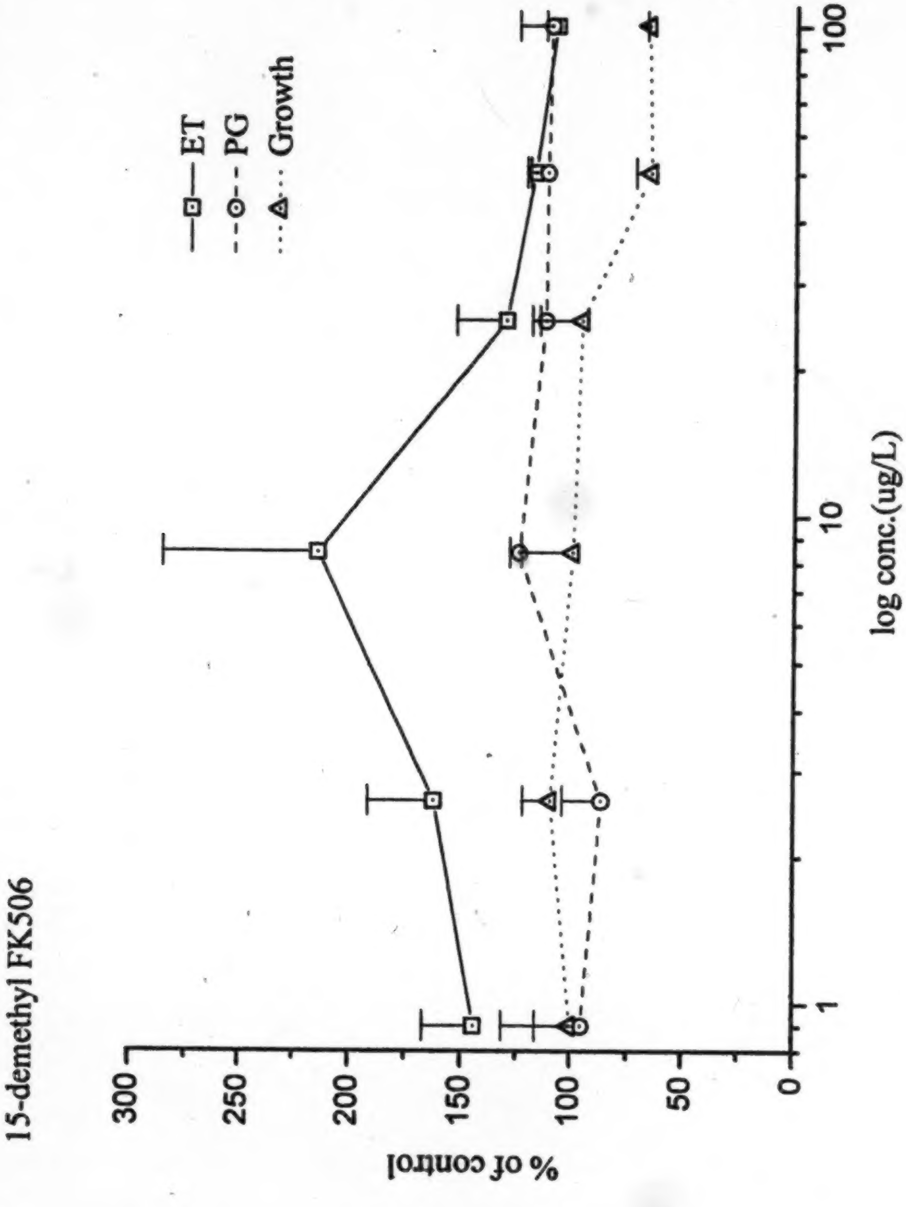


Figure IV-1 E: The influence of 15-demethyl FK506 on the release of endothelin- 1, 2 and prostaglandin F1α, and the growth rate of the cell culture.

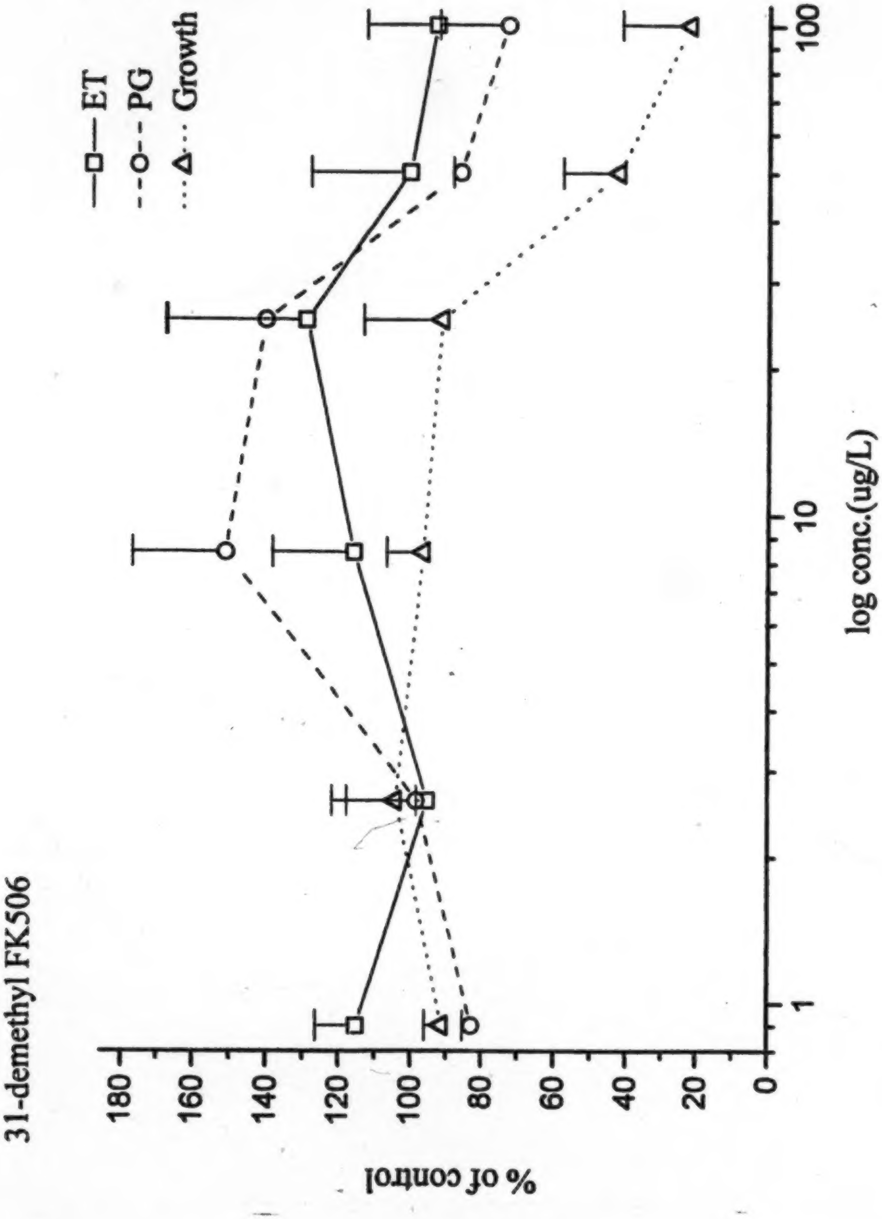


Figure IV-1 F: The influence of 31–demethyl FK506 on the release of endothelin- 1, 2 and prostaglandin F1α, and the growth rate of the cell culture.

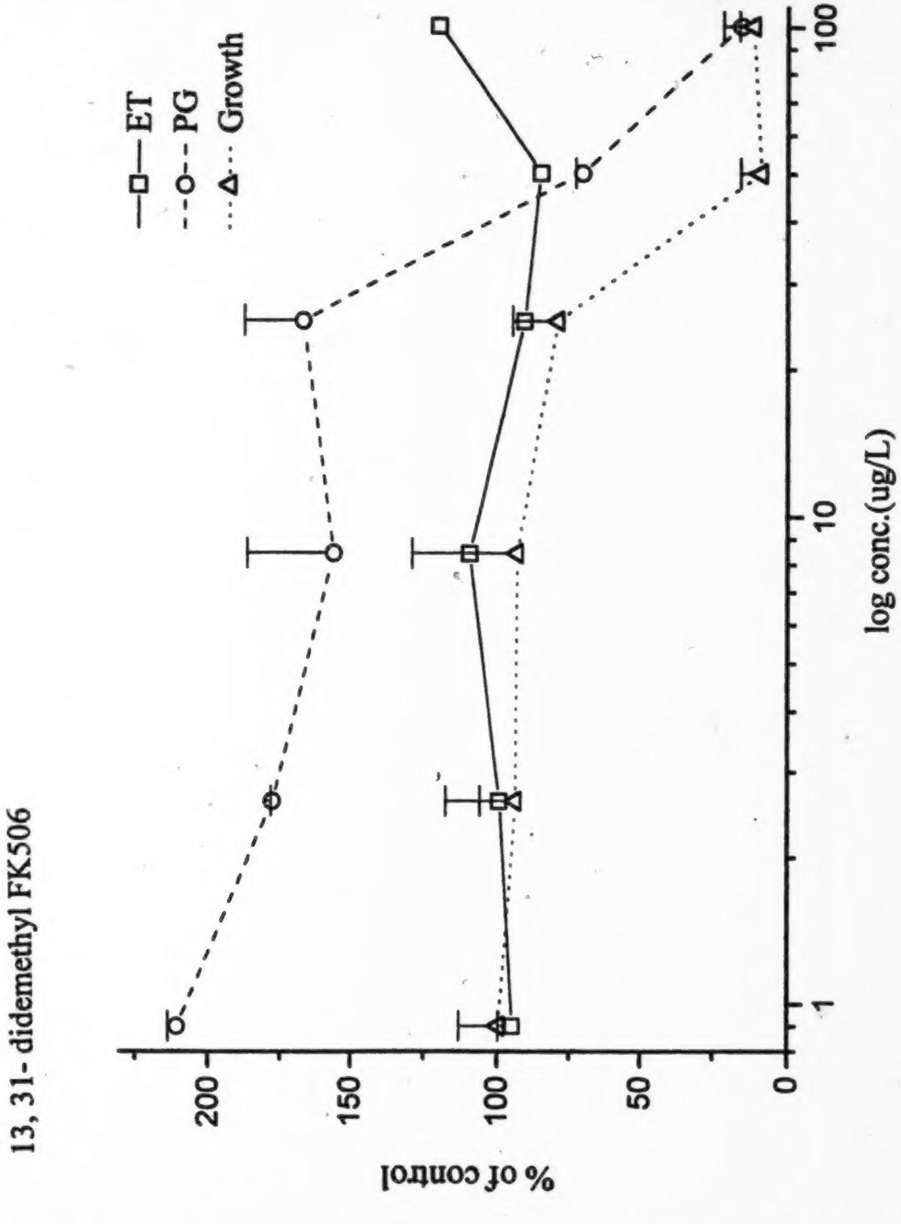


Figure IV-1 G: The influence of 13, 31–didemethyl FK506 on the release of endothelin- 1, 2 and prostaglandin F1α, and the growth rate of the cell culture.

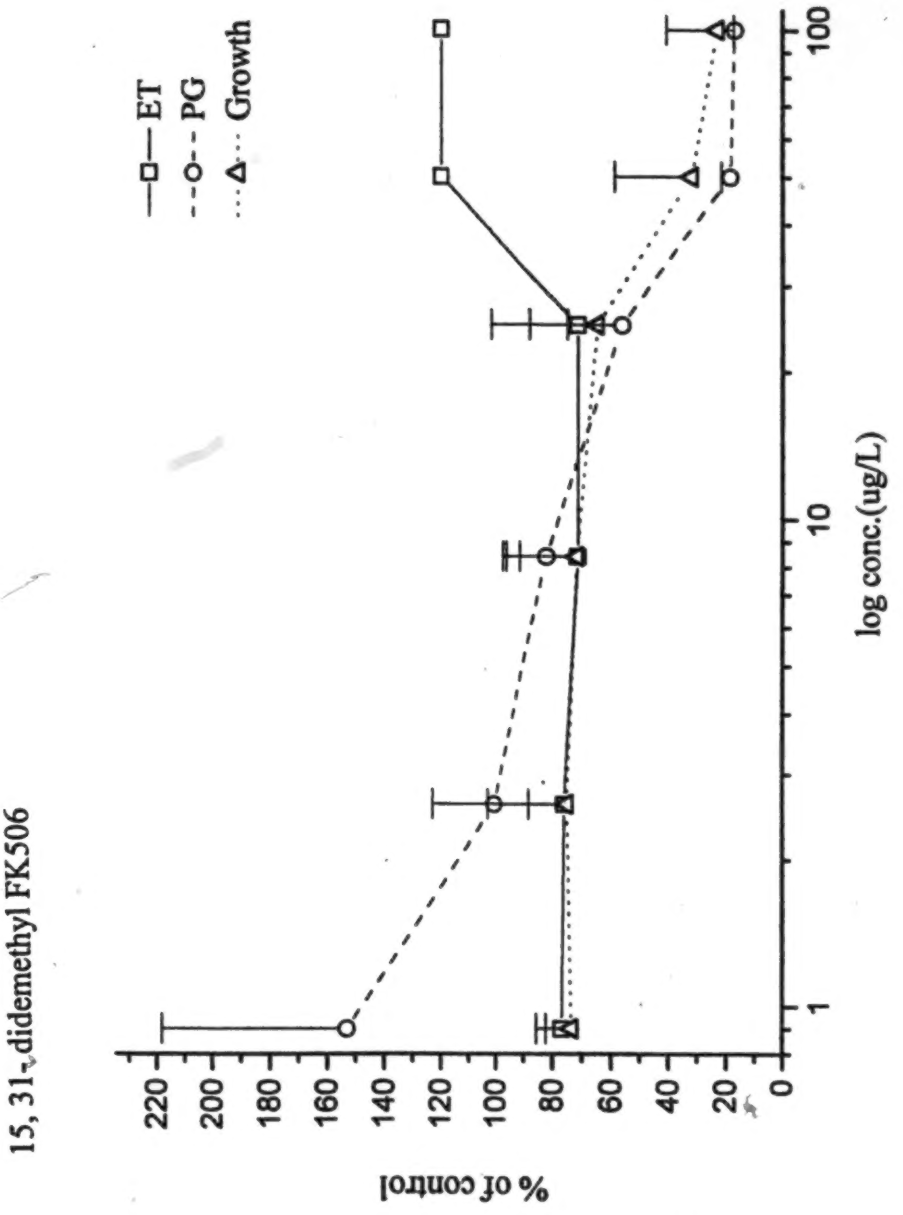


Figure IV-1 H: The influence of 15, 31-didemethyl FK506 on the release of endothelin- 1, 2 and prostaglandin F1α, and the growth rate of the cell culture.

# Chapter V

## Discussion

### Identification of FK506 Metabolites

The phase I metabolite patterns of FK506 in pooled human whole blood, pooled human urine, a rabbit liver microsome metabolic system, and a microbial culture were evaluated (Figure II-4). The possibility of phase II FK506 metabolites in human pooled urine was also evaluated (Table II-1). Our colleagues in the laboratory evaluated and confirmed the chemical structures of several major FK506 metabolites (13-demethylated FK506, 15-demethylated FK506, 31-demethylated FK506, 13,15-didemethylated FK506, 13,31-didemethylated FK506, and 15,31-didemethylated FK506) (data not shown). All identifiable FK506 metabolites in human whole blood can be produced by rabbit liver microsomes in different proportions. Rabbit liver microsomes and the microbial culture produce more FK506 metabolites than those identified in human biological fluids. The rabbit liver microsomal system is a good source of large quantities of FK506 metabolites for further study because it not only produces hydrophobic FK506 metabolites such as 31-demethyl FK506 but also produces FK506 metabolites such as demethyl-hydroxy FK506 that are more hydrophilic. Most of the hydrophobic metabolites can be identified in FK506 patients' specimens. It is necessary to harvest the hydrophilic metabolites because these metabolites may be produced by FK506-treated patients whose liver enzyme activity has been induced. It was noted that a major FK506 metabolite, 31-demethylated FK506, found in the rabbit liver microsomal metabolic system and the microbial culture is absent from human specimens. The proportion of 13-demethylated FK506 to FK506 is higher in human pooled urine than in pooled blood. This may result from the effect of concentration by the kidney. Dihydroxy-FK506 can be identified in pooled human urine but not in the pooled human blood.

From the study of possible phase II FK506 metabolites in urine, there was no evidence that they exist. However, pooled FK506 patient urine was used in the study. It is possible that the phase II FK506 metabolites do exist in individual patients and have been averaged out in the pooled urine sample. Möller et al. (1999) stated that urinary excretion of FK506 metabolites only accounted for less than 3% of total administered dose and that FK506 metabolites are released in the bile. Thus, feces maybe a better source in which to study phase II FK506 metabolites.

Seven FK506 metabolites have been previously identified. These metabolites include 13-demethylated FK506, 15-demethylated FK506, 31-demethylated FK506, 13,15-didemethylated FK506, 13,31-didemethylated FK506, 15,31-didemethylated FK506, and 12- hydroxy FK506 (Iwasaki et al., 1993; Iwasaki et al., 1995). These workers investigated the biological functions of these metabolites (which will be discussed in later section), but they did not report the steady state concentrations of these metabolites in human trough blood. Gonschior and Christians (Gonschior et al., 1996) have reported the metabolite patterns in blood from liver and kidney transplant patients. They found that the levels of didemethyl and didemethyl-hydroxy FK506 were increased in individuals with impaired liver function, but it was concluded that the levels of these metabolites were not high enough to significantly influence the biological functions of the drug and the cross-reactivity in the immunoassays. However, their results did not distinguish the positional isomers such as 13-demethyl vs. 15-demethyl FK506. In addition, their results showed a pattern of more hydrophilic metabolites than in my study, possibly due to different extraction procedures used (solid-liquid vs. liquid-liquid).

### Therapeutic Monitoring of FK506

An ideal drug should have a predictable, easily measurable dose-dependent effect, a broad therapeutic range, and uncomplicated kinetics that allow a standardized dosage regimen. However, this ideal situation is usually not achieved for the most drugs, and in some situations therapeutic drug monitoring (TDM) is advocated to provide information about the adequacy of the dosage regimen or the likelihood of toxicity.

Drugs that need to be therapeutically monitored usually fall into one or more of the following categories: 1. They show a narrow therapeutic window, meaning toxic symptoms occur at the concentrations close to those that show full therapeutic effects; 2. They have large intra- and inter-individual variabilities in pharmacokinetics; 3. There are factors that interact with drug pharmacokinetics or pharmacodynamics; and 4. Therapeutic failure may occur on standard dosing (Lindhelm, et al, 1995). FK506 fits into all of these categories. Thus, it is necessary to set up a TDM program for patients under FK506 therapy. There are several considerations when setting up the program: 1) the use of specific and sensitive analytical methods, 2) identification of which matrix should be used to avoid artifacts, e. g. the partition difference between matrix components, 3) identification of major metabolites, and 4) proper assessment of the concentration-effect relationship (Jusko et al., 1995; Alak, 1997). After initial failures with plasma monitoring of FK506, the current recommendation is to monitor FK506 in trough whole blood. There are several advantages to monitoring trough blood FK506 levels: 1) it gives a reliable and reproducible measure of the minimum mean steady state concentration and can be considered to reflect drug elimination; 2) minimum effective concentrations for immunosuppressants can be proposed; 3) it may be performed daily and in outpatients; and 4) it is the most documented monitoring method. But with only monitoring FK506 trough blood concentrations, the concentration-effect relationship may be weak and few pharmacokinetic data can be obtained. One of the objectives of this thesis is to compare different methods used for routine therapeutic monitoring of FK506.

LC/MS analysis proved to be a good reference assay to measure FK506 trough blood concentrations. The between-run precision and accuracy of the LC/MS assay are summarized in table III- 2. The C.V.s for FK506 were also low with percentages ranging from 3.8% to 4.6%. The analytical recovery obtained for the LQC (7.5 μg/L), MQC (15 μg/L), and HQC (25 μg/L) are 92.4%, 95.2% and 92.8% respectively. The within-run precision and accuracy of the LC/MS assay are summarized in table III- 3. As with the between-run studies, within-run precision and accuracy were determined using a total of six replicates of the LQC, MQC, and HQC whose concentrations were determined from one calibration curve. The C.V.s ranged from 4.0 % to 5.9 %. The analytical recovery obtained for the QCs were 94.6%, 95.8%, and 97.7% respectively. The coefficient of variations (CV) for different comparisons was all less than 10%.

Two commercial test kits are available for routine monitoring of FK506. Both kits utilize the same monoclonal anti-FK506 antibody. The quantitation limits are 2 μg/L for the IMx FK506 micro-particle enzyme immunoassay (MEIA), and 0.3 μg/L for the Pro-Trac FK506 enzyme- linked immunosorbent assay (ELISA). In order to assess the specificity of the MEIA and ELISA methods, various comparative studies with LC/MS have been conducted in this thesis (results shown in figure III- 2, figure III- 3, figure III- 4, and figure III- 5). From the comparison of FK506 trough blood concentrations measured between LC/MS and ELISA, the equation of linear regression is as follows: ELISA= 0.39 + 0.9997 × LC/MS. From the comparison of FK506 trough blood concentrations measured between MEIA and ELISA, the equation of linear regression is as follows: ELISA= -0.11 + 0.86 × MEIA. It can be deducted from the two equations above and the results shown in figure III- 3 and figure III- 5 that both ELISA and MEIA have positive intercepts compared with the concentrations measured by LC/MS. From the bias plotting (figure III- 3 and figure III- 5), it is clear that both ELISA and MEIA have positive bias compared with LC/MS assay. The correlation between LC/MS and ELISA, and MEIA and ELISA is very similar with r =0.96 and r = 0.94, respectively. It was noted that ELISA tends to yield higher results than LC/MS does, with 3.86% positive bias; while MEIA tends to yield higher results than ELISA does, with 17.57% positive bias.

From earlier studies done by other scientists, it was not clear whether both immunoassays are suitable for routine monitoring of FK506. Some studies stated that both immunoassays can be used as methods for routine therapeutic monitoring of FK506 (Alak, 1997; Brunet et al., 1998; Jusko, 1995; Gonschior et al., 1996; Winkler et al., 1996). The correlation between both immunoassays and LC/MS ranged from 0.71 to 0.89 (r). However, some studies did show an unavoidable positive bias existing in the results measured by immunoassays compared with the results done by LC/MS or LC/MS/MS (Gonschior et al., 1996; Winkler et al., 1996; Taylor et al., 1996; Zhang et al., 1997; Murhty et al., 1997; Salm et al., 1997; MacFarlane et al., 1999). Taylor et al. (1996) found a positive bias of 17% using an in-house ELISA when they compared the concentrations of FK506 measured in a group of liver transplant recipients to those obtained with LC/MS/MS. Zhang et al. (1997) compared the MEIA I to LC/MS/MS and found a positive bias of 3% with a correlation coefficient of 0.96. Both Murhty et al. (1997) and Salm et al. (1997) have shown that ELISA yields consistently lower concentrations of FK506 than the MEIA method in patient specimens. In a more recent study, MacFarlane et al. (1999) discovered that a positive mean bias of 2.5 μg/L between ELISA and LC/MS/MS came from a group of FK506-treated patients with impaired liver function. 71% of these samples agreed within 3 μg/L while 3% exhibited a difference of over 10 μg/L. On the other hand, a positive mean bias of only 0.6 μg/L was shown in the comparison of ELISA and LC/MS/MS from a group of patients with normal liver function. From this recent study and our study, it is clear that while a small subset of patients with cholestasis may require closer evaluation with a more specific methodology, the majority of the FK506-treated patients may be satisfactorily monitored with ELISA. As for MEIA, it needs further investigation to see if the positive bias comes from a subset of patients or not.

The intra-assay and inter-assay CV of ELISA range from 5.6% to 25.5% and the assay is linear over the range 0.3 to 30 μg/L. The CV for MEIA range from 8.1% to 11.8% (Alak, 1997). The recommended FK506 trough blood concentrations measured by MEIA are 10-15 μg/L for the initial stage and 5-15 μg/L for the maintenance period (Shaw et al., 1999). The positive bias seen with some FK506 patient blood samples measured by ELISA and MEIA is presumably due to the production of several FK506 metabolites that can cross-react with the monoclonal anti-FK506 antibody used in these immunoassays. In the following section, the significance of most identifiable FK506 metabolites, including their steady state concentrations in patients' blood, their cross-reactivity with anti-FK506 monoclonal antibody, their immunosuppressive activity, and their ability to cause renal dysfunction will be discussed.

## Biological Significance of FK506 Metabolites

### Metabolite patterns and trough blood steady state concentrations

The steady state concentrations of identifiable FK506 metabolites in 20 human blood samples were measured (Table IV-1). Only four FK506 metabolites, 13-demethylated FK506, 15-demethylated FK506, 13,15-didemethylated FK506, and one of the demethyl-hydroxy- FK506 species, are consistently identified in individual human whole blood samples. Didemethyl-hydroxy- and hydroxy- FK506 are occasionally identified in individual human blood specimens. Of the metabolites, 13-demethylated FK506 has the highest steady state concentration and is about one seventh of that of FK506. The total amount of FK506 metabolites is about one- third of the concentration of FK506 in human whole blood. Similar experiments have been conducted in Gonschior and Christian's laboratory (Christians et al., 1991; Christians et al., 1992; Gonschior et al., 1996), with the metabolites adding up to 43% of total FK506 in human whole blood. This is about 10% higher than the results from our study. The possible reasons for the differences may come from different extraction procedures or different analytical methods. They used solid-liquid extraction instead of liquid-liquid extraction as we did. Furthermore, there is no distinguishing of the positional isomers of the same metabolites such as 13-demethyl FK506 vs. 15-demethyl FK506 from their results. It is very important to distinguish the subspecies because they may have different biological significance. From the results obtained in our experiments, several questions can be raised. Which FK506 metabolite(s) will have the ability of immunosuppression or cross-reacitivity with anti-FK506 in the analytical immuno-assays? Will the FK506 metabolites have significant influence on the toxicity of the drug? Several experiments were designed to seek out the answers. The results will be discussed in the following sections.

## Immunosuppressive activity

The immunosuppressive activity of FK506 metabolites was evaluated by the mixed lymphocyte reaction. The principle of this assay is to mix and culture lymphocytes from two different individuals and observe the immuno-reactions occurring during the culture. The induced lymphocytes proliferate and absorb $^3$H-thymidine during the process of proliferation. The growth of the lymphocytes is measured by counting the radioactivity of the cells that absorb $^3$H-thymidine. The radioactivity will decrease if inhibition of immuno-response occurs. The results are recorded in Table IV- 1. The major pharmacological mechanism of FK506 is believed to be the inhibition of calcineurin (CN). A comparison was also done for FK506 and its metabolites between the effect on the mixed lymphocyte reaction and the inhibition of CN to see if there is a correlation. The results are shown in Table IV- 2.

31-Demethylated FK506 has very similar potency of immunosuppression to that of FK506. The immunosuppressive activity of 13-demethylated FK506 and 13,31-didemethylated FK506 are about 20% of the parent drug. 15-demethylated FK506, 15, 31-didemethylated FK506, and one demethyl-hydroxy FK506 that can be identified in human blood showed less than 10% of the immunosuppressive activity of the parent drug. Comparing the results of the mixed lymphocyte reaction and the inhibition of CN activity, the results of the percentage inhibition of CN from peripheral blood mononuclear cells (PBMC) correlate well with the percentage inhibition of the mixed lymphocyte reaction except in the case of 13,31-didemethylated FK506. Moreover, the percentage of inhibition of CN from whole blood is quite different from the results of the mixed lymphocyte reaction, especially for 13-demethylated FK506 and 13,31-didemethylated FK506. 13-demethylated FK506 inhibits the activity of CN to a greater degree in whole blood than in PBMCs. This may result from the fact that the components such as drug-binding protein that can influence the inhibition of CN in lysed blood are more abundant than the ones in lysed PBMCs. It may also result from various mechanisms involving the immuno-response for the mixed lymphocyte reaction; that is, the inhibition of the immuno-reaction of induced lymphocytes is not only dependent on the inhibition of CN but also depends on other unknown mechanisms. Generally speaking, the FK506 metabolites that show more ability to inhibit the mixed lymphocyte reaction (MLR) also inhibit CN activity to a greater extent.

Other researchers have also investigated the immunosuppressive activity (MLR) of several major FK506 metabolites (Iwasaki et al., 1993; Iwasaki et al., 1995; Tamura et al., 1994). Their results are summarized in table V- 1. With seven metabolites (13-demethyl FK506, 15-demethyl FK506, 31-demethyl FK506, 13,15-didemethyl FK506, 13,31-didemethyl FK506, 15,31-didemethyl FK506, and 12-hydroxy FK506) identified, there is no difference between our results for the immunosuppressive activity of these FK506 metabolites and theirs. 31-Demethyl FK506 exhibited about 100% immunosuppression compared with the parent drug. The rest of the FK506 metabolites exhibited less than 25% immunosuppression relative to the parent drug. One paper mentioned the inhibition of CN activity by FK506 the $IC_{50}$ of FK506 was 1 nM (Fruman et al., 1992), which is similar to our results ($IC_{50}$= 0.9 nM).

### Cross-reactivity

The FK506 ELISA kit was used to examine the cross-reactivity of FK506 metabolites with the anti-FK506 antibody used in this immunoassay. Each metabolite was tested three times. 31-demethylated FK506 exhibited about 100% cross-reactivity, while 15-demethylated FK506 and 15,31-didemethylated FK506 exhibited approximately 90% cross-reactivity. Other FK506 metabolites showed less than 10% cross-reactivity with the antibody.

From the results of the measurement of the steady state concentrations of FK506 metabolites (Table IV-1) and the cross-reactivity of the metabolites, 31-demethylated FK506 could be significant in influencing routine therapeutic monitoring of FK506. It shows 100% cross-reactivity with the anti-FK506 in the immunoassay but it normally cannot be identified in human blood and urine. 31-

demethylated FK506 may be produced if the enzymatic system of metabolism becomes abnormal such as in the case of dysfunction or induction by other drugs. When this happens, the existence of 31-demethylated FK506 may influence the results of therapeutic monitoring of FK506, and under-estimation of the dosage could occur. 15-demethylated FK506 and 15,31-didemethylated FK506 are also important because they show about 90% cross-reactivity and exist in patients' blood samples.

Other researchers have also investigated the cross-reactivity of several major FK506 metabolites (Iwasaki et al., 1993; Iwasaki et al., 1995; Tamura et al., 1994). Their results are summarized in table V- 1. Generally speaking, there is no difference between our results for the cross-reactivity of these major FK506 metabolites in the immunoassays and theirs.

Once the metabolic system becomes induced, these metabolites may be over-produced. Such an increase in levels of the metabolites will influence the concentration of parent drug (FK506) measured by routine immunoassays. Further investigations should be designed to focus on patients with liver abnormalities and see if they produce more of the FK506 metabolites mentioned above. This will prove if the immunoassays are suitable for different groups of FK506 patients.

## Influence on the release of vasoactive substances from endothelial cells

There are several major side effects of FK506, with renal dysfunction being the most frequently observed. Though little is known about the mechanism of FK506 nephrotoxicity, available data from several studies regarding renal dysfunction (Scharschmidt et al., 1983; Brenner et al., 1986) and the toxicity of cyclosporine (Copeland et al., 1992) suggest that it may be associated with a disturbance in renal hemodynamics, possibly brought about by alterations in the production of vasoactive substances such as endothelin-1 and prostacyclin. Some researchers have used cultures of rat primary renal mesangial cells in an *in vitro* study to investigate the influence of FK506 on the release of endothelin-1 from the cells (Goodall et al., 1995). The results did show that FK506 caused a significant dose-related increase in endothelin-1 release. The peak increase of the release of endothelin was when the concentration of free FK506 reached 8 μg/L. In other words, increases in the secretion of endothelin and decreases in the production of prostacyclin should be related to the nephrotoxicity. Renal mesangial cells (renal inter-capillary cells) belong to a type of kidney smooth muscle cells (Essig et al., 1996). Within a renal micro-environment, other types of cells such as endothelial cells from the renal blood capillary can also release vasoactive substances that influence the filtration rate of the kidney (de Mattos et al., 2000). Thus, primary pig aorta endothelial cells were chosen as representative of vascular endothelial cells. They were cultivated for the purpose of preliminarily examining the influence of FK506 and its metabolites on the release of endothelin-1 and prostacyclin. The results are shown in Figure IV-1.

The influence of a series of concentrations (1, 2.5, 8.5, 25, 50, 100 µg/L) of free FK506 and metabolites on the release of endothelin-1 and prostacyclin from the culture of primary aorta endothelial cells and on the cellular growth rate were investigated. The last two concentrations are significantly higher than the respective trough concentrations found in blood of transplant patients. However, the intracellular and extracellular concentrations of the drug and metabolites may be considerably different. We did not quantitate intracellular concentrations of metabolites in our study. From my results, 31-demethylated FK506 and two species of didemethylated FK506 significantly decreased the growth rate of the endothelial cells at the concentrations of 50 and 100 µg/L. FK506 and other metabolites did not have the same effect on the growth rate of the cells. The cells that show lower growth rates may either stay in a certain stage of proliferation or go through cytotoxicity and die, but this hypothesis needs further verification. FK506 and 15-demethylated FK506 showed very similar patterns in the concentration-related stimulation of endothelin release. The peak secretion occurred at a free drug concentration of 8.5 µg/L or between 8.5 and 25 µg/L. The other FK506 metabolites did not have any influence on the release of endothelin from pig aorta endothelial cells. The original hypothesis stated that the decrease of glomerular filtration rate by FK506 may be caused by the increases of endothelin or the decreases of prostacyclin. Opposing the original hypothesis, 13,31-didemethylated FK506 showed the ability to induce the secretion of prostacyclin, a vasodilator, in a dose-related manner. This will counter the effect of endothelin on the glomerular filtration rate, thus protecting against nephrotoxicity.

There are several possibilities to explain the results obtained. First, the time interval for harvesting the supernatant of the cellular culture was 24 hours. This time interval may be too long so that the endothelial cells have been activated by the release of endothelin and other stimulators. The activation of endothelial cells will lead to the production of eicosanoids such as prostaglandin $E_2$, prostacyclin (prostaglandin $I_2$), and thromboxane $A_2$ (Bustos et al., 1997). The establishment of this time interval was based on the experience of a similar experiment done for cyclosporine (Copeland et al., 1992). This criterion may not be suitable for FK506. Further experimentation should be designed to focus on choosing different culture intervals to see if there is any difference in the release of endothelin and prostacyclin. Secondly, there are vasoactive substances other than endothelin and prostacyclin that can influence the hemodynamic environment of the kidney. Nitric oxide (NO) and thromboxane $A_2$ ($TxA_2$) are among them (Bustos et al., 1997). The overall influence on the glomerular filtration rate will be the result of effects contributed from various factors. It is difficult to perform a simple *in vitro* experiment that covers all the factors. An *in vivo* experiment to mimic the biological environment should be designed to cover all possible vasoactive substances and also different cultivating time intervals. Another possible way to study nephrotoxicity for FK506 and its metabolites is to analyze their effects on the contraction of a segment of aorta blood vessels. In this way, the overall effects of the release of complex vasoactive substances can be counted and the measurement can be simply given in a mathematical way.

## Biological significance

Some researchers have published data on immunosuppression and cross-reactivity with anti-FK506 antibody in immunoassays for several FK506 metabolites (13-demethylated FK506, 15-demethylated FK506, 31-demethylated FK506, 13,15-didemethylated FK506, 13,31-didemethylated FK506, 15,31-didemethylated FK506, and one subspecies of hydroxylated FK506), and their results were similar to ours (Iwasaki et al., 1993; Iwasaki et al., 1995; Tamura et al., 1994; Alak, 1997). 31-Demethyl FK506 exhibited around 100% immunosuppression and cross-reactivity, while 15-demethyl FK506 exhibited around 90% cross-reactivity. But these investigators did not study the rest of the FK506 metabolites and they did not report the steady state concentration of each metabolite. In order to present the significance of FK506 metabolites in a numerical manner, an equation named the "Clinical Significance Index (CSI)" was devised (Langman, et al., 1996). The equation is as follows:

$$\text{CSI} = \text{steady state conc} \times \text{immunosuppression} \times \text{cross-reactivity} \times \text{nephrotoxicity}$$

The value of each component for FK506 is equal to 1. The values of the rest metabolites are calculated relative to the ones for FK506.

This original equation was found to be of little use because of the complexity of the measurement for nephrotoxicity. If we ignore the nephrotoxicity portion of the equation, the preliminary CSI values for FK506 metabolites can be calculated and are shown in Table V-2. It is interesting to note that this preliminary CSI values for most of the metabolites are 0 or close to 0. One of the components in the equation for the metabolites is equal to 0, causing the value of CSI to become 0. To probe further, the steady state concentrations measured here are only the concentrations in trough blood samples. Their values cannot be represented as the concentrations in different tissues. The proportions of the parent drug to the metabolites may also change from tissue to tissue. All these possibilities made it impossible to present the significance of FK506 metabolites in a single number. Therefore, we will discuss the significance of FK506 metabolites according to their performance in various experiments.

Based on the x-ray structure of the $Ca^{2+}$-Calmodulin-Calcineurin-FK506-FKBP12 complex (Griffith et al., 1995; Itoh et al., 1995; Kissinger et al., 1995), the region of $C_{13}$ through $C_{21}$ of FK506 is inserted into the binding area. The majority of contacts made by FK506 are from $C_{15}$-$C_{17}$ and at the $C_{21}$ allyl group. This means that alterations of this region that lead to a loss of binding may result in the alteration of the immunosuppressive ability of FK506. This has been proven by the results from the inhibition of immunoreactivities observed in the mixed lymphocyte reaction. 31-Demethylated FK506 does not lose the ability of immunosuppression while 13-demethylated FK506 and 15-demethylated FK506 lose part or all of the immunosuppressive ability compared with parent drug. Although there is no evidence that 31-demethylated FK506 exists in human specimens (blood and urine), the importance of this metabolite should not be ignored. A human liver microsome metabolic system should be used in an experiment to see if 31-demethylated FK506 can be produced or not. If it can be produced, that means that there is a possibility that 31-demethylated FK506 would reside extravascularly in the tissue because of its hydrophobicity so that we cannot identify it in blood or urine specimens. 31-Demethylated FK506 would thus become very significant in the overall immunosuppression. Therapeutic monitoring of FK506 only may not be sufficient to correlate the effects of the drug with immunosuppression. Or in other situations, 31-demethylated FK506 may be easily metabolized further so it can not be identified in blood or urine samples. Further investigation is needed to prove this hypothesis.

According to the results obtained for the cross-reactivity of FK506 metabolites with anti-FK506 antibody used in the commercially available immunoassays, the epitope that the antibody was directed against was the region around $C_{13}$ of FK506. It would be better to design an antibody against the $C_{13}$-$C_{15}$ region of FK506 because there is no detectable 31-demethylated FK506 existing in human blood to cross-react with the antibody. 13-Demethylated and 15-demethylated FK506 will not cross-react with anti-FK506 that is designed against the $C_{13}$-$C_{15}$ region. This improved antibody used in the analytical immunoassays would benefit the therapeutic monitoring of FK506.

The results from the study of nephrotoxicity are more complicated. The original hypothesis was unproven. There are other factors, such as NO and $TxA_2$ (Bustos et al., 1997), that should be taken into account when measuring the influence of FK506 metabolites on the glomerular filtration rate. Unlike the results from the measurement of immunosuppression and cross-reactivity, the position of $C_{31}$ of FK506 is not as important in nephrotoxicity as in the studies of immunosuppression and cross-reactivity with anti-FK506. 15-Demethylated FK506 has similar effects to FK506 on the release of endothelin. The region around $C_{15}$-$C_{17}$ of FK506 is the contact area for FKBP12 and CN. The alteration around this area should lead to the changes in the behavior of the drug. It is obvious that this did not happen regarding the effect of the metabolites on the release of endothelin. The involvement of other FKBPs such as FKBP51 and FKBP52 (Renoir et al., 1990; Barent et al., 1998), or of other mechanisms, may be important in this situation. 31-Demethylated FK506, 13,31-didemethylated FK506, and 15,31-didemethylated FK506 have the ability to decrease the growth rate of the endothelial cells. It means that in this case, the alteration of $C_{31}$ is of biological significance. It leads to the implication of an unknown mechanism that can decrease the growth rate of the endothelial cells. We did not design an experiment to inspect whether the decrease of the growth rate is because the cells stop in a certain stage of the cell growth cycle or because the cells go through cytotoxicity and die. A couple of investigations have shown that FK506 can induce apoptosis of mouse brain capillary endothelial cells and enhance apoptosis of antigen-stimulated peripheral T lymphocytes (Kochi et al., 2000; Migita et al., 1999). This will be a direction for future study.

## Possible Directions for Further Investigation

There are three major possible directions for further investigation according to the results obtained in this study. The first one is related to renal toxicity. In order to examine the influence of FK506 and its metabolites on the renal toxicity, the vasoconstriction of a segment of rat aorta should be investigated in a newly designed experiment. The influence of the combination of released vasoactive substances on the endothelial and muscle cells can be measured using a simple method. The isometric tension from a segment of pre-dilated rat thoracic aorta could be measured with a transducer attached to the tissue bath computer. The constriction elicited by various concentrations of FK506 or its metabolites can be expressed as a percentage of the maximal dilatation increase produced by the pre-dilatation agent. In this way, the overall capacity of FK506 or its metabolites to constrict blood vessels can be measured without consideration of different factors involved. Epstein et al. (1997) did a very similar experiment for cyclosporine and FK506 with renal and coronary artery smooth muscle contraction. The results showed that FK506 could not induce the contraction. However, the concentration of FK506 they used was too high, 500 to $5 \times 10^6$ μg/L, probably not representing the true clinical situation. The concentrations should cover the therapeutic range of FK506 in the trough human blood. There have been very few studies done to investigate the distribution of FK506 in various tissues. Only one paper reported the distribution of FK506 in a mouse model (Yokogawa et al., 1999). The tissue concentrations of FK506 in normal mice were in the following order: lung > spleen > kidney > heart > gut > brain > liver. The highest tissue-to-blood concentration ratio of FK506 was 10.6 (lung) while the lowest was 1.84 (liver). The tissue concentrations of FK506 were presented in ng/g wet tissue and using 0.5 to 5000 µg/L of FK506 should cover the range of tissue concentrations. However, there is no report discussing the distribution of FK506 metabolites in tissues. By performing the above experiments, the CSI may regain its utilization for the purpose of presenting the significance of FK506 metabolites because the renal toxicity can be calculated in numbers. Moreover, if the cell cultures of mesangial cells and vasoendothelial cells are still preferred, the original experiment should be optimized. The time interval for the cell culture and all of the vasoactive factors involved should be considered. It will also be beneficial if an *in vivo* experiment can be established to study the influence of FK506 and its metabolites on renal toxicity. A study of glomerular filtration rate and morphological changes of the kidney from an animal model that mimics the human biological environment of renal dysfunction should provide insight into the mechanisms of renal toxicity.

The second direction is to focus on the other mechanisms of how FK506 is involved in nonimmune cells. This can be divided into two parts; one is related to FKBPs, and the other to calcium release channels. In addition to FKBP12, several different FKBPs have been identified recently. FKBP51/FKBP52 are believed to be associated with the complex of heat shock protein 70/90 (Bruner et al., 1997). Heat shock proteins are important in protein folding and assembly (Bresnick et al., 1989; Diehl et al., 1993; Hutchison et al., 1994). They may also have functions in immunoregulatory processes that are not yet fully understood. It has been shown that several heat shock proteins of various species can modulate functions in macrophage immunity by directly increasing cytokine production. The relationship between the roles of heat shock proteins, IL-1, IL-6, ET, and prostacyclin has been drawn in Figure V-1 (based on the information from Agui et al., 1994; Chen et al., 1994; D'Souza et al., 1994; Vajo et al., 1996; Bustos et al., 1997; Odoux et al., 1997; Yokoo et al., 1997). Based on this scheme and the results obtained from the experiment on renal toxicity, we believe that other types of FKBPs are important in non-lymphoid cells when FK506 or its metabolites are involved. Recently, FK506 has been found to suppress IL- 6 and NO expression in bacterial wall-induced polyarthritis in rats (Fuseler et al., 2000). This supports my hypothesis of the involvement of FKBP51/FKBP52 in FK506-related side effects.

Recently, FKBP12 has been found to be tightly associated with the calcium release channel (ryanodine receptor) on the membrane of sarcoplasmic reticulum (Timerman et al., 1993; Brillantes et al., 1994) and with the inositol 1,4,5- triphosphate receptor ($IP_3R$) on the membrane of endoplasmic reticulum (Cameron et al., 1997). The ryanodine receptor is a large intracellular $Ca^{2+}$ release channel required for excitation-contraction coupling in striated muscles, including skeletal and cardiac muscles. It is also expressed in a wide variety of tissues such as brain and endothelial cells (Ogawa et al., 1994). $IP_3R$ and FKBP12 form a complex. When $IP_3R$ is activated by $IP_3$, $Ca^{2+}$ is released from the lumen of the endoplasmic reticulum and results in increases in local $Ca^{2+}$ concentrations. This increase activates protein kinase C (PKC), which phosphorylates the $IP_3R$ and further increases $IP_3$- mediated $Ca^{2+}$ flux. Increased $Ca^{2+}$ levels also activate calcineurin/calmodulin, causing an association of this complex with $IP_3R$ /FKBP12 and activation of the phosphatase activity of CN. The activated CN dephosphorylates the phosphorylation site on the $IP_3R$ done by PKC and decreases $Ca^{2+}$ flux. FK506 can reverse the stabilizing effects of FKBP12 on the rynodine receptor, and it can displace FKBP-12 and CN from $IP_3R$, resulting in suboptimal subunit cooperation and leaky $Ca^{2+}$ channels. Hence, this may also be a possible mechanism involved in FK506 toxicity. Experiments designed to verify the hypotheses of the involvement of FKBP12/ryanodine receptor, FKBP12/$IP_3R$ and the FKBP51/52/heat shock protein70/90 will be useful to help understand the role of FK506 and its metabolites in biological systems.

The third direction for future investigation is to focus on better drug therapy for transplant patients, possibly based on the structure of some of the metabolites. Although different types of immunosuppressive therapy have been developed, not one of them is completely effective. Chronic rejection still occurs even if the patients are requiring optimal therapeutic drug monitoring (Nagano et al., 1997). There are many reasons, but the strength of the biological system cannot be ignored. When one side of the biological system loses its balance, the other side of the system will try to maintain the normal function. In other words, if we try to suppress one side of the immune system in order to lower the risk of rejection in transplantation, the other side of the biological system will try to bring back its original function in order to keep the individual functional. So many times, toxicity or chronic rejection occurs. This is an old philosophy from Chinese medicine, but it is very true. Therefore, the clinical application of immune tolerance becomes more and more popular because in this way patients will not lose their immune response to fight other diseases and their biological system will not lose its balance. Although the mechanisms responsible for immune tolerance are still not fully understood, the earliest studies suggested that the prior engraftment of haematopoietic cells of donor origin, under the right conditions, could lead to a state in which a subsequent organ allograft from the donor could be accepted without additional immunosuppression (Platt, 1998). In the process of immune tolerance, there is a stage at which the donor haematopoietic cells are being accepted by the recipients as their own cells. This requires cytotoxic drugs or irradiation that may produce complications. Since some FK506 metabolites may lead to a decrease of cellular growth rate (possible cytotoxicity), they may be good agents for promoting the acceptance of donor haematopoietic cells by selectively inhibiting alloreactive T cells in an antigen-specific manner. The use of a combination of FK506 with other immunosuppressive drugs that allow the development of immunoregulatory T cells may ultimately merge the fields of immunosuppression and immune tolerance.

## Conclusions

Therapeutic monitoring is recommended when a drug has a narrow therapeutic index and is used chronically. It is difficult to establish a suitable therapeutic range for FK506 because there are no simple parameters for the assessment of its immunosuppressive effect. Moreover, the lack of such criteria and the substantial intra- and inter-individual variation in FK506 pharmacokinetics are strong arguments for drug monitoring to prevent over- or under-immunosuppression. Recommendations and guidelines for therapeutic monitoring of FK506 have been established (Jusko, 1995; Mcmaster et al., 1995, Ueda et al., 1995). The target range are according to the highest percentage of successful transplantation rate. There may still be various complications even if patients' trough blood concentrations are kept within the therapeutic range. The guidelines also state that only if the FK506 metabolites are significant should they be monitored.

An important factor to consider when monitoring FK506 is the effect of the metabolites. The metabolites can contribute to immunosuppression, cross-reactivity with the antibody used in the analytical immunoassays, and toxic effects. The immunosuppression and the cross-reactivity with the antibody used in the immunoassays are dependent on the concentrations of FK506 metabolites. The overall clinical significance of each FK506 metabolite, expressed as a function of immunosuppressive activity, cross-reactivity, and steady state concentration, is markedly less than that of the parent drug. Some metabolites may show significantly high values for certain factors, but they contribute minimally to overall immunosuppression and cross-reactivity. Based on the data presented here, the parent drug alone should be monitored in therapeutic drug monitoring in normal situations. The metabolites that show significance in the factors mentioned above should not be ignored when problems related to the monitoring occur, as, for example, when the dose/trough blood concentration does not fall into the target range.

A number of approaches can be used to assess the appropriateness of the dosing regimen for immunosuppressive drugs. The first one involves the assessment of clinical response. This approach has its limitations since signs of rejection or toxicity may be difficult to recognize clinically. The second one involves the assessment of pharmacokinetic properties of the drug and relating various parameters (trough levels, area under the curve, etc.) to immunosuppressive efficacy or toxicity. This approach also has its limitations since the concentrations of the drug measured may not reflect the pharmacologically active concentration due to cross-reactivity of inactive metabolites in the assays. In addition, therapeutic ranges for the drugs have been difficult to establish due to dependency on the type of transplant, time post-transplant, and concomitant immunosuppressive drugs. The third one, a pharmacodynamic approach, involves measurement of the biological effect of the drug and adjustments of dosage to optimize immunosuppression and minimize side effects. This approach may have significant advantages over the measurement of drug concentrations, especially in multiple drug therapy where the assessment of a suitable therapeutic range may be difficult. The immunosuppression of FK506 and its metabolites shown in the mixed lymphocyte reaction correlated well with the results of the inhibition of CN in PBMCs. The drug is routinely used in combination with other immunosuppressive agents most of the time, thus posing more difficulty in establishment of its therapeutic range. Pharmacodynamic monitoring of FK506 may be possible by measuring the inhibition of CN.

As the spectrum of immunosuppressive agents available for use in transplantation increases in the future, establishment of therapeutic ranges for the drugs will become more difficult. Factors such as drug combinations, types of transplantation, fractions of drug measured (total vs. free), and the time post-transplant will have an impact on this range. The measurement of the biological response to drugs may provide a viable alternative to traditional therapeutic drug monitoring by assessing the state of immunosuppression.

| Metabolite | Cross-Reactivity | Immunosuppressive activity |
|---|---|---|
| tacrolimus | 100% | 100% |
| 13-demethyl tacrolimus | 0% | 10% |
| 15-demethyl tacrolimus | 90% | 0.1 % |
| 31-demethyl tacrolimus | 109% | 60-100% |
| | | |
| 15,31-didemethyl tacrolimus | 92% | 0% |
| 13,31-didemethyl tacrolimus | 0% | 1% |
| 13,15-didemethyl tacrolimus | 0% | 0% |
| | | |
| 12-hydroxy tacrolimus | 9% | 3% |
| didemethylhydroxy tacrolimus | 15% | ?? |

Table V- 1: Published cross-reactivities and immunosuppression of metabolites of FK506. Values obtained from Iwasaki et al. (1993, 1995).

| FK506 metabolites | Human blood level | Immuno-suppression | Antibody cross-reactivity | CSI |
|---|---|---|---|---|
| *FK506 | 1 | 1 | 1 | 1 |
| *13-demethyl | 0.15 | 0.2 | 0 | 0 |
| *15-demethyl | 0.07 | 0 | 0.9 | 0 |
| 31-demethyl | 0 | 0.98 | >1 | 0 |
| *13,15-didemethyl | 0 | 0 | 0.05 | 0 |
| *13,31-didemethyl | 0.03 | 0.23 | 0.03 | 0.0002 |
| 15,31-didemethyl | 0 | 0.07 | 0.91 | 0 |
| Dm-hydroxy (1) | 0 | 0.03 | 0.03 | 0 |
| Dm-hydroxy (2) | 0 | 0.01 | 0.01 | 0 |
| Dm-hydroxy (3) | 0 | 0.13 | 0.01 | 0 |
| *Dm-hydroxy (4) | 0.02 | 0.04 | 0.02 | 0.00016 |
| Dm-hydroxy (5) | 0 | 0.2 | 0.08 | 0 |
| Didm-hydroxy | 0 | 0.01 | 0.03 | 0 |
| Didm-trihydroxy | 0 | 0.01 | 0.01 | 0 |
| Hydroxy (1) | 0 | 0 | 0 | 0 |
| *Hydroxy (2) | 0 | 0.07 | 0.18 | 0 |
| *Hydroxy (3) | 0 | 0 | 0 | 0 |

Table V-2: Preliminary Clinical Significance Index (CSI} of FK506 and its metabolites
Dm = demethyl.

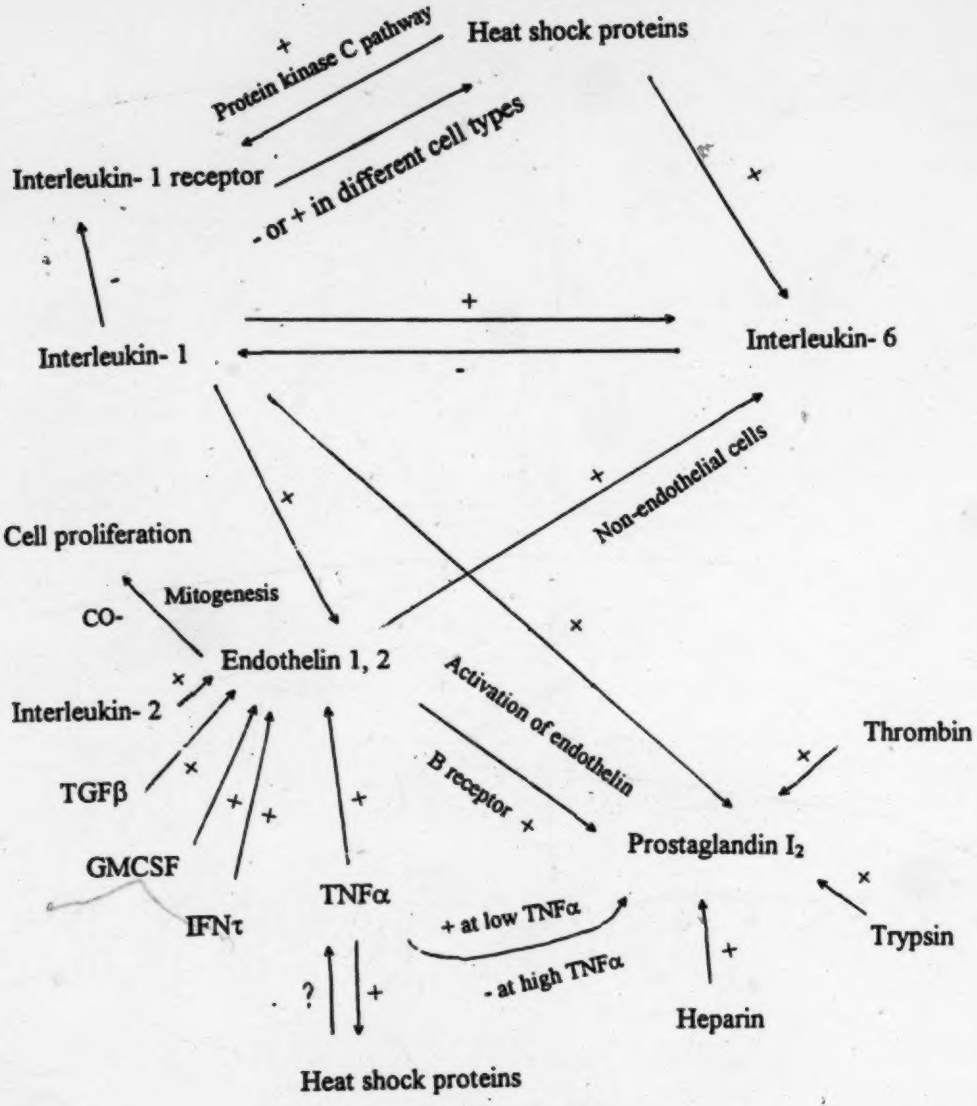

Heat shock proteins
+
Protein kinase C pathway
Interleukin- 1 receptor
- or + in different cell types
+
-
+
Interleukin- 1
-
Interleukin- 6
+
+
Non-endothelial cells
Cell proliferation
Mitogenesis
CO-
Endothelin 1, 2
+
×
Activation of endothelin
Interleukin- 2
×
Thrombin
×
TGFβ
B receptor
+
+
+
+
Prostaglandin $I_2$
GMCSF
TNFα
+ at low TNFα
×
IFNτ
Trypsin
?
+
- at high TNFα
+
Heparin
Heat shock proteins

Figure V- 1: The flow chart of the relationship between heat shock protein, interleukin-1, interleukin- 6, endothelin-1, 2, and prostaglandin $I_2$.

+: stimulates   -: inhibits
GSF: granulocyte stimulation factor
TGFβ: β transforming growth factor β
GMCSF: granulocyte monocyte colony-stimulation factor
IFNι: ι interferon
TNFα: α tumor necrosis factor

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